THE CROSSTALK BETWEEN O-GLCNACYLATION AND PHOSPHORYLATION IN INSULIN SIGNALING

by
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ABSTRACT

Extensive crosstalk between phosphorylation and O-GlcNAcylation is involved in regulating insulin signaling. In addition, many of the key regulators of insulin signaling have been shown to be O-GlcNAcylated. In this study, we first explored the crosstalk between phosphorylation and O-GlcNAcylation globally, and then focused on an upstream signaling regulator key to insulin signaling, insulin receptor substrate 1, IRS1. Through regulating cellular O-GlcNAcylation levels in differentiated adipocyte 3T3-L1 cells by specific OGT and OGA inhibitors, we measured the affects of O-GlcNAcylation on the phosphorylation status of key molecules. We observed that O-GlcNAcylation regulates the flux through the two main divergent cellular pathways of insulin signaling, one regulating glucose metabolism and the other regulating cellular proliferation via MAP kinase. Decreased O-GlcNAcylation was associated with increased glucose utilization, and increased O-GlcNAcylation was associated with increased insulin-induced cellular proliferation pathways. Since IRS1 is early in the insulin signaling pathway and is both highly O-GlcNAcylated and highly phosphorylated, we focused on the crosstalk of those two PTMs on IRS1. The phosphorylation spectrum of IRS1 revealed by 2D electrophoretic analyses, partially explained why decreased O-GlcNAcylation facilitated glucose uptake. Two new O-GlcNAcylation sites were mapped through CID/ETD mass spectrometry. With help of protein truncation and site mutagenesis techniques, we propose a mechanism suggesting that the O-GlcNAcylation of Ser635, may be a negative regulator of the C-terminal O-GlcNAcylations on IRS1. Since C-terminal O-GlcNAcylation of IRS1 inhibits binding with

PI3K, Ser635 O-GlcNAcylation, unlike O-GlcNAcylation at other sites on IRS1, is thus an activator for PI3K/AKT signaling.

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LIST OF ABBREVIATIONS

2-DE……..two dimensional gel electrophoresis

2-DG……2-deoxy glucose

AD……..Alzheimer's disease

AMPK……AMP-activated protein kinase

ATP……adenosine triphosphate

BEMAD……β-elimination followed by Michael Addition with DTT

CAD……..collision activated dissociation

CaMKIV……calcium/calmodulin-dependent kinase IV

CARM1……coactivator-associated arginine methyltransferase 1

C/EBP……..CCAAT/enhancer binding protein

CID……..….collision induced dissociation

CIP……calf intestinal phosphatase

CTD……..carboxyl terminal domain

DEX……...dexamethasone

DTT……dithiothreitol

ECD……..electron capture dissociation

eNOS……..endothelial nitric oxide synthase

ER……..estrogen receptor

ERK……..extracellular signal regulated kinase

ES……..embryonic stem

ETD…….electron transfer dissociation

FoxO1…….Forkhead box O1

FT-ICR…….Fourier transform ion cyclotron resonance

GAB1……..GRB2 associated binding protein 1

GABA……...……gamma-aminobutyric acid

GalT……galactosyltransferse

GFAT……..…l-glutamine:d-fructose-6-phosphate aminotransferase

GRB2…….growth factor receptor bound protein 2

GRIF……GABAA receptor-associated protein

GSK……..glycogen synthase kinase

HAT……histone acetyltransferase

HBP…….…….hexosamine biosynthetic pathway

hOGA…….human OGA

Hsp…….heat shock protein

IBMX…….3-isobutyl-1-methylxanthine

IKKβ……Iκ kinase β

IR……insulin receptor

IRS1…….insulin receptor substrate 1

iTRAQ……..isobaric tags for relative and absolute quantification

kDa…….kilodalton

HRP……horseradish peroxidase

MAPK……mitogen activated protein kinase

MEK……mitogen activated protein kinase kinase

MYPT1……..…..myosin phosphatase targeting subunit 1

NF……neurofilament

O-GlcNAc ……O-linked β-N-acetylglucosamine

OGA ……..….β-D-N-acetylglucosaminidase

OGT……O-linked β-N-acetylglucosaminyl transferase

OA……..okadaic acid

PC-biotin……..photocleavable biotin

PDB……protein data bank

PEST……Pro, Glu, Ser and Thr

PGC-1α……..peroxisome proliferator activated receptor γ coactivator-1α

PHF-tau……...paired helical filamentous tau

PI3K……phosphatidylinositol-4,5-bisphosphate-3-kinase

PIP3……phosphatidylinositol 3,4,5-triphosphate

PKA…….protein kinase A

P/S……penicillin/strptomycin

PTM……..…posttranslational modification

PUGNAc……

……..O-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-N-phenylcarbamate

QUIC-tag……..quantitative isotopic and chemoenzymatic tag

RNAP II …….RNA polymerase

RNAi…….RNA interference

RP-HPLC……reverse phase high performance liquid chromatography

SILAC…….……stable isotope labeling with amino acids in cell culture

SOS……..son of sevenless

sWGA…….succinylated Wheat-Germ Agglutinin

TAFII110……TATA-binding protein-associated factor

TAS…….tagging-via-substrate

TFIID…….transcription factor II D

TMG……..Thiamet G

TMT……tandem mass tags

TNF……..….tumor necrosis factor

TPR…….....tetratricopeptide repeats

UDP-GlcNAc ……uridine diphospho-N-acetylglucosamine

UDP-GlcNAz……UDP-N-azidoacetylglucosamine

CHAPTER ONE

SITE-SPECIFIC INTERPLAY BETWEEN O-GLCNACYLATION AND PHOSPHORYLATION IN CELLULAR REGULATION

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ABSTRACT

O-linked β-N-acetylglucosamine (O-GlcNAc) modification of serine/threonine residues on nuclear and cytoplasmic proteins is a ubiquitious, rapidly cycling monosaccharide. Extensive crosstalk or interplay exists between O-GlcNAcylation and phosphorylation, which regulates signaling in response to nutrients and stress. Since its discovery, difficulties in detecting and in mapping specific O-GlcNAc sites have limited progress in this field. In the past five years, the development of novel O-GlcNAc detection and enrichment methods, combined with advances in mass spectrometry, have significantly improved our understanding of the functions of O-GlcNAcylation. Global site mapping studies have revealed O-GlcNAc's many interactions with phosphorylation-mediated signaling. Despite their intimate and dynamic interplay, the mechanisms regulating O-GlcNAcylation and phosphorylation are quite different. Phosphorylation is catalyzed by hundreds of distinct genetically encoded kinases, each with its own catalytic activity. In contrast, in mammals uridine diphospho-N-acetylgucosamine:polypeptide β-N-acetylglucosamyl transferase (OGT) is encoded by a single highly-conserved X-linked gene. However, like RNA polymerase II (RNAP II) and phosphatases, OGT's protein specificity and targeting are determined by its transient associations with many other regulatory subunits to create a multitude of specific holoenzymes. O-GlcNAcase (OGA), the enzyme that removes O-GlcNAc, is also encoded by a single highly conserved gene, and is targeted to its substrates in a manner similar to OGT. The extensive crosstalk between O-GlcNAcylation and phosphorylation represents a new paradigm for cellular signaling.

INTRODUCTION

Ser(Thr)-O-linked β-N-acetylglucosamine (O-GlcNAc) is a common posttranslational modification (PTM) of nuclear and cytosolic proteins. O-GlcNAcylation was serendipitously discovered in experiments designed to detect terminal GlcNAc residues on the surfaces of living lymphocytes, but surprisingly nearly all of the O-GlcNAc was found to be on nucleocytoplasmic proteins, where dogma said protein glycosylation was absent [1,2] . O-GlcNAcylation occurs in some bacteria, many protozoa, viruses, filamentous fungi and in all metazoans, including plants, but thus far has not been definitively documented in yeast. O-GlcNAcylation is distinguished from "classical" glycosylation in the following respects: 1) It is nearly exclusively on cytoplasmic and nuclear proteins, compared to the extracellular and luminal localization for most other protein-bound glycosyl moieties. 2) O-GlcNAc is generally not modified or elongated to more complex structures. 3) Like phosphorylation, O-GlcNAcylation occurs at substoichiometric amounts and rapidly cycles on and off polypeptides at different rates at specific sites in response to different physiological stimuli, such as hormones, growth factors, and mitogens[3-6] (more in review[7]).

Uridine diphospho-N-acetylgucosamine:polypeptide β-N-acetylglucosamyl transferase (OGT) and β-D-N-acetylgucosaminidase (OGA) are the paired enzymes responsible for the addition and removal of O-GlcNAc, respectively. Although O-GlcNAcylation has many properties similar to phosphorylation, the two post-translational modifications are regulated very differently. Phosphorylation is determined by hundreds of specific kinases, each encoded by a distinct gene. In contrast, both OGT and OGA are encoded by a single highly conserved gene in animals (two alleles of OGT in plants [8].),

which contains their catalytic centers and numerous protein-protein interaction domains [9-11]. Like RNAP II, phosphatases [12] and other enzymes, OGT's and OGA's protein specificity is mostly determined by its transient interactions with many binding partners to form many substrate specific holoenzymes [13,14]. The OGT catalytic subunit also has pronounced specificity to peptide sequence around the attachment site, but no absolute consensus sequence exists. In addition, OGT's enzymatic activity is highly responsive to the concentration of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), the donor substrate for O-GlcNAcylation [15].

OGT is found in many organisms, from *C. elegans*, *Drosophila, plants* to human, and shares very high homology among species [7]. The *Ogt* gene resides on the X chromosome near the centromere. Knock-out studies have shown that *Ogt is* required for the viability of embryonic stem (ES) cells and other cells, even in cell culture [16]. Tissue targeted *Ogt* mutations in mice and several cell lines, such as T cell, neuronal cell, fibroblasts, using the Cre-loxP recombination system demonstrated that loss of O-GlcNAcylation results in concomitant loss of cell function and eventually cell death [16]. In contrast to plants and mammals, in *C. elegans*, *Ogt* knockout, while producing profound metabolic changes, such as insulin resistance and abnormal metabolism, does not cause the death of the worm. These and other genetic studies support a role for O-GlcNAcylation in cellular signaling and insulin resistance [17]. For humans, there are at least three transcript isoforms of OGT: 110 kilodalton (kDa) (p110), 78kDa (p78) and 103kDa (p103), with variation from 2.5-13.5 tetratricopeptide repeats (TPRs). The catalytic p110 subunit can form either a homodimer or heterotrimer with p78 in the cytoplasm and nucleus depending on cell type, while p103 is localized to the mitochondria [7,9,18]. OGT's catalytic subunit is

composed of two separate domains. Its N-terminus contains TPRs, which mediate protein-protein interactions, thus functioning in OGT substrate recognition and multimerization. The C-terminus of p110 has the catalytic domain, possessing glycosyltransferase activity [19,20]. Structural analysis of 11.5 of N-terminus TPR domain shows a right-handed superhelix of about 100 Å long and 35Å wide, which likely provides for substrate binding on its concave surface [21]. The crystal structure of a bacterial orthologue of human OGT (hOGT), from *Xanthomonas campestris* OGT (*Xc*OGT) shows that OGT has a conserved UDP-GlcNAc binding pocket. This structure also shows that the last few TPRs are, closely associated with C-terminal active site, forming an elongated, continuous groove from the donor substrate binding site to the end of the TPR superhelix [22,23]. Molecular modeling of the bacterial structure and the mammalian TPR-repeat structure strongly supports the structure proposed for the mammalian OGT [13-15].

OGA was initially identified as hexosaminidase C, with β-linked GlcNAc substrate specificity, neutral pH optima, and mainly a cytosolic localization, unlike lysosomal hexosaminidases [24-26]. Strikingly, *Oga* knockouts in *C.elegans* showed similar metabolic defects as the *ogt* knockouts, such as elevated stores of glycogen, and decreased lipid storage. This finding suggests that O-GlcNAc cycling rates and not its absolute stoichiometry may be important in its regulation of cellular metabolism. Human OGA (hOGA) is a 92 kDa bifunctional protein, its N-terminus contains O-GlcNAc hydrolase activity, and its C-terminus bears a putative histone acetyltransferase (HAT) domain. Caspase-3, a cysteine-aspartic protease that serves as the "executioner protease" in apoptosis, cleaves these two domains during apoptosis without affecting the O-GlcNAc hydrolase catalytic activity [27,28]. hOGA catalyzes O-GlcNAc hydrolysis using a substrate-assisted mechanism, which

requires the involvement of the 2-acetamido group of GlcNAc as the nucleophile [29]. Similar catalytic mechanisms were also confirmed on bacterial OGA orthologues from *Clostridium perfringens* NagJ and *B. thetaiotaomicron* BtGH84 [30,31].

O-GLCNACYLATION IS A NUTRIENT AND STRESS SENSOR

As stated above, UDP-GlcNAc, the immediate donor substrate for O-GlcNAcylation, is a terminal product of the hexosamine biosynthetic pathway (HBP) (Figure 1). This sugar nucleotide is second only to adenosine triphosphate (ATP) in abundance of high energy small molecules in cells. When HBP flux changes, UDP-GlcNAc levels also change rapidly, altering the extent of O-GlcNAcylation of many proteins. HBP flux and UDP-GlcNAc availability are affected directly by many different nutrients, such as glucose, fatty acids, and amino acids. Exogenously added glucosamine is efficiently 'salvaged' by cells, and small amounts can dramatically increase UDP-GlcNAc pools in cells. When glucose enters cells, besides being used for glycogen synthesis or glycolysis, a portion (2-5%) is converted to UDP-GlcNAc [32]. Free fatty acids also increase HBP flux by inhibiting glycolysis, resulting in elevated fructose-6-phosphate levels. Acetyl-CoA, produced by fatty acid metabolism, serves as the donor for the acetylation of glucosamine in the formation of UDP-GlcNAc. L-glutamine: D-fructose-6-phosphate aminotransferase (GFAT) is the key rate-limiting enzyme of the HBP. GFAT converts fructose-6-phosphate to glucosamine-6-phosphate, using glutamine as the nitrogen donor. GFAT is by passed by glucosamine, which enters directly into the HBP by being phosphorylated to form glucosamine-6-phosphate [33]. *Ob/ob* mice, which lack of leptin, a hormone that regulates nutrient uptake, have elevated GFAT activity

and increased UDP-GlcNAc levels in muscle. Leptin mRNA and protein expression are upregulated by high levels of nutrients. UDP-GlcNAc levels are increased in tissues by hyperglycemia, hyperlipidaemia, as well as by glucosamine [34,35].

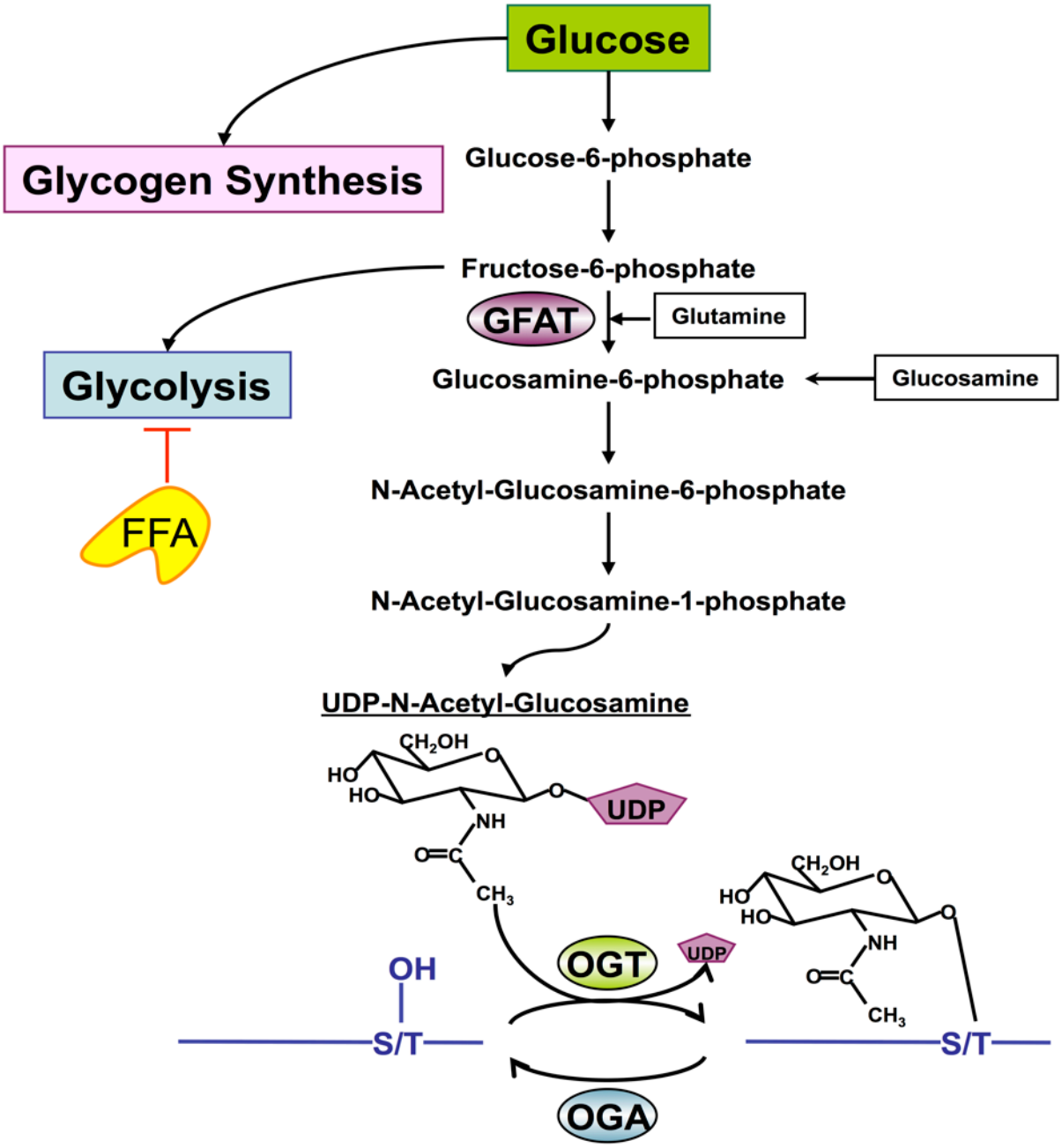

Figure 1. HBP and O-GlcNAcylation. In addition to glycolysis and glycogen synthesis, 2-5% of cellular glucose is processed through HBP. GFAT mediated conversion of fructose-6-phosphate to glucosamine-6-phosphate is the rate-limiting step of HBP. The presence of elevated FFA has been linked to inhibition of glycolysis and elevated glucose flux through HBP. UDP-GlcNAc is the end product of the HBP. OGT catalyzes the transfer of O-GlcNAc from UDP-GlcNAc onto the hydroxyl moiety of serine or threonine residues,while OGA catalyzes the removal of the sugar. Addition of glutamine or glucosamine elevates global O-GlcNAcylation.

The substrate specificity of OGT, as well as its enzymatic activity are regulated by different levels of UDP-GlcNAc [15]. In hyperglycemia, elevated HBP flux cause increased O-GlcNAcylation on specific insulin signaling molecules, such as Akt, insulin receptor substrate 1 (IRS1), and others, to negatively regulate the insulin pathway. Excessive O-GlcNAcylation of insulin signaling molecules results in less insulin-stimulated glucose transport [33].

Hyperglycemia and hyperlipidemia also affect transcription by increasing the O-GlcNAcylation of transcription factors, such as Sp1. For example, the saturated fatty acid, palmitate, which causes increased HBP flux, increases Sp1's DNA binding activity, as well as its transcription activity by elevating its O-GlcNAcylation state [36].

O-GlcNAcylation is also a sensor of cellular stress. Multiple forms of stress increase glucose uptake and thus HBP flux and concomitantly increase UDP-GlcNAc concentrations, leading to rapid increased global O-GlcNAcylation. Heat shock protein 70 (Hsp-70), a key chaperone protein, which protects cells from stress, is an endogenous O-GlcNAc binding lectin [37]. Many different stresses, such as oxidative, osmotic, UV, ethanol, and heat shock induce rapid and global increased O-GlcNAcylation. Increased O-GlcNAcylation induced by thermal stress occurs rapidly and returns back to basal levels after removing the stress. Thermal stress increases OGT's enzymatic activity but does not increase its expression. In contrast, OGT expression is increased by ethanol treatment or by osmotic stress. Ogt knockout through Cre-LoxP in mouse embryonic fibroblasts cells (MEFs) or knockdown of OGT by using RNA interference (RNAi) in mouse neuroblastoma cells (Neuro-2A) both decreased thermotolerance. Artifically increasing cellular O-GlcNAc levels by pharmacological inhibiting OGA activity, using the inhibitor, O-(2-acetamido-2-deoxy-D-

glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc), resulted in increased thermotolerance. This protective effect of O-GlcNAcylation on thermal stress is partially explained by elevating Hsp70 and Hsp40 expression [38], chaperon complex of these two proteins is well known as heat protector in mammalian cells [39].

Paradoxically, despite lower UDP-GlcNAc and ATP levels, global O-GlcNAcylation is strikingly increased when neuronal cells are deprived of glucose. When Neuro-2a cells are deprived of glucose, increased OGT mRNA and protein expression were observed. OGT's targeting to a population of substrate proteins, rather than an increase in its catalytic activity was found. OGT's increased activity towards this glucose-deprivation dependent population of proteins in Neuro2a cells was dependent upon AMP-activated protein kinase (AMPK) and mitogen-activated protein kinase (MAPK)/p38 [13]. However, in human hepatocellular carcinoma cells (HepG2), glucose-deprivation dependent elevation of O-GlcNAcylation results mainly from increased OGT mRNA and protein expression, as well as decreased OGA expression, while not kinase activity dependent [40,41]. Glucose deprivation increases O-GlcNAcylation in human non-small cell lung carcinoma A549 cells, by yet a different mechanism. In A549 cancer cells, glucose deprivation results in increased HBP flux as well as increased UDP-GlcNAc levels. The source of glucose driving the increased HBP flux is from increased glycogen phosphorylase activity, the rate-limiting step in mobilizing glycogen. In A549 cancer cells glucose deprivation also results in increased GFAT activity. In addition, glucose deprivation in A549 results in higher OGT enzymatic activity and lower enzymatic activity for OGA without changing OGT and OGA expression levels [42]. The different mechanisms elevating O-GlcNAcylation in response to glucose

deprivation may result from the distinctly different mechanisms regulating energy metabolism in these different cell-types. Thus, there are complex and intimate relationships between nutrient status, stress status and O-GlcNAcylation. O-GlcNAcylation not only responds to cell nutrient and stress status, but also regulates the cell's reactions to variations in nutrients and stress.

DETECTION AND SITE MAPPING OF O-GLCNAC

O-GlcNAcylation can be detected by using pan-specific anti-O-GlcNAc antibodies (CTD 110.6, RL2, HGAC 85) or lectins (succinylated Wheat-Germ Agglutinin, sWGA). CTD 110.6 is an IgM monoclonal antibody produced using chemically synthesized O-GlcNAcylated peptide based upon a single repeat of the RNAP II carboxyl terminal domain (CTD) motif as the antigen [34]. RL2 is an IgG monoclonal produced using nuclear pore protein complex fractions as the antigen. HGAC 85 is a monoclonal antibody made against streptococcal group A polysaccharide, which recognizes O-GlcNAc [43]. All of these antibodies bind with relatively low affinity and favor the detection of highly abundant O-GlcNAcylated proteins or those with clustered O-GlcNAc moieties. While mostly recognizing O-GlcNAc, these antibodies differ in their dependency upon peptide structure and bind different subsets of O-GlcNAcylated proteins [44,45]. Recently, several O-GlcNAc-peptide specific monoclonal antibodies have been described, which may prove valuable in further defining the biological roles of O-GlcNAcylation [46]. The lectin, sWGA, is able to recognize any terminal GlcNAc, including those on N-glycans, O-glycans or O-GlcNAc. Regardless of which probe is used, additional controls, such as inhibition of binding by

excess GlcNAc, prior treatment with hexosaminidase, or 'capping' the epitope with galactosyltransferase should be used [34] to validate specificity.

As with any post-translational modification, mapping the attachment site(s) is a prerequisite toward understanding the modification's biological functions. Site mapping O-GlcNAcylation is very difficult due to its special characteristics: 1) O-GlcNAcylation usually has low stoichiometry at each site on proteins. For example, stoichiometry of O-GlcNAcylation on α-crystallin, a structural protein in the eye lens, is less than 2% [47,48]. 2) In mass spectrometry, the presence of even a small proportion of unmodified peptide ions severely suppresses ionization of O-GlcNAcylated peptides. 3) The β-linkage between O-GlcNAc moiety and Ser/Thr is labile. Upon fragmentation by standard collision induced dissociation (CID), the glycosidic bond breaks first, resulting in poor fragmentation of the glycopeptide and loss of any site information. With the development of sample enrichment methods and new mass spectrometry (MS) fragmentation methods, such as electron capture dissociation (ECD) or electron transfer dissociation (ETD), it is now possible to map and quantify both O-GlcNAc and phospho-sites simultaneously in complicated samples using relatively limited amounts of protein [49].

EARLY APPROCHES TO MAP O-GLCNAC SITES

In early studies, mapping O-GlcNAc sites was a tedious and time-consuming process, often taking nearly one-year. In these early studies, O-GlcNAc site mapping was achieved by first tagging the O-GlcNAc protein or peptide by enzymatically attaching a [^{3}H]-galatose, using bovine milk β-1,4-galactosyltransferse (GalT1) [1]. Multiple rounds of reverse phase high performance liquid chromatography (RP-HPLC) of tryptic peptides were then used to

obtain pure radiolabeled glycopeptides. Automated Edman degradation was used to sequence the peptide, and manual Edman degradation was used to determine which residue contained the radioactivity [50]. Several O-GlcNAc sites were mapped in this way, such as Thr^{48} and Thr^{431} on neurofilament M (NF-M), Thr^{21} and Ser^{27} on NF-L [51,52]. Sites on estrogen receptor, as well as synapsin I were also mapped via this method [53-55]. However, this method had many problems: 1) It contains too many steps and requires expensive radioactive sugar nucleotide. 2) Many rounds of HPLC and Edman degradation are needed to definitively identify the major O-GlcNAc sites on a single protein. 3) The overall procedure was time consuming and difficult. 4) Large amounts of start sample were required. For example, in order to map O-GlcNAc sites, at least 10 picomole of pure, radiolabeled glycopeptide was required. Due to sample loss, low stoichiometry of O-GlcNAcylation as well as limits in detecting [^{3}H]-galatose, typically nanomole to even micromole amounts of starting protein were often required.

MAPPING O-GLCNAC SITES ON RECOMBINANT OR IN VITRO EXPRESSED PROTEINS

It often is difficult to obtain enough of a low abundance endogenous regulatory protein, such as a transcription factor, to map O-GlcNAc sites, even with the best methods currently available. Three approaches have proven useful, at least as a starting point: 1) For low abundance proteins where DNA is available, the protein can be transcribed and translated in a reticulocyte lysate system, which efficiently O-GlcNAcylates nascent proteins [56]. Lectin affinity chromatography on sWGA is then used to detect O-GlcNAcylation [55,57,58]. 2) Overexpression of the protein in E. coli, which does not O-GlcNAcylate

proteins, allows for production of a polypeptide substrate, which can be O-GlcNAcylated *in vitro*, using recombinant OGT. This approach allows for site-mapping, which can readily be confirmed *in vivo* by site-directed mutagenesis, combined with anti-O-GlcNAc Western blotting. 3) Fairly large quantities of *in vivo* O-GlcNAcylated proteins can be obtained by over-expressing affinity-tagged proteins (eg. His, Flag, or glutathione S-transferase(GST))). This can be done in either insect cells via baculoviral infection [53,55,59-61] or transfection in HEK293 mammalian cells [62-64]. Thus far, sites used for O-GlcNAcylation in these model systems are also used in other cell-types. However, putative O-GlcNAc sites need to be directly confirmed in the cell type under study, usually via site-directed mutagenesis, or by mass spectrometry.

O-GLCNAC ENRICHMENT METHODS

Using large-scale protein preparation, while mitigating the low stoichiometry of O-GlcNAc increases the risk of ion-suppression by non-glycosylated peptides. Therefore, it is almost always necessary to purify O-GlcNAcylated proteins or peptides away from the unmodified peptides.

IMMUNOAFFINITY/LECTIN BASED ENRICHED METHODS

The simplest O-GlcNAc enrichment approach is to purify O-GlcNAcylated proteins using immunoaffinity or lectin chromatography. O-GlcNAcylated proteins from cell lysates are efficiently enriched by both anti-O-GlcNAc antibody affinity chromatography or by sWGA affinity chromatography [65-67]. However, both methods favor enrichment of high

abundance proteins (eg. nuclear pore proteins) or those with multiple clustered O-GlcNAc residues. Low abundance proteins with single or widely separated O-GlcNAc sites are difficult to purify using these tools and their binding to the affinity solid support is often competed by more abundant or higher affinity O-GlcNAcylated proteins.

METABOLIC IN VIVO TAGGING OF O-GLCNAC FOR ENRICHEMENT (TAGGING-VIA-SUBSTRATE: TAS)

This enrichment method takes advantage of the fact that the cellular machinery will take up and use N-azidoacetylglucosamine (GlcNAz) as an alternative substrate to GlcNAc. In order to facilitate uptake by living cells, GlcNAz is peracetylated. Upon uptake, the peracetylated GlcNAz is rapidly deacetylated by intracellular esterases. GlcNAz enters the HBP just like glucosamine, but at lower efficiency, and is converted into UDP-N-azidoacetylglucosamine (UDP-GlcNAz). UDP-GlcNAz is utilized by OGT, and O-GlcNAz is attached to Ser/Thr sites. The GlcNAz on proteins enables conjugation via Staudinger ligation to enrichment tags, such as a biotinylated phosphine [68]. After ligation, the labeled sample can be selectively purified using strepavidin/avidin beads. This method was termed "tagging-via-substrate approach" (TAS). By using this method, 199 putative O-GlcNAcylated proteins were identified in Hela cells (human cervical cancer cell line) through nano-HPLC-MS/MS [69]. The high affinity between biotin and avidin/streptavidin allows for a high degree of enrichment by allowing stringent washes to eliminate nonspecifically bound material. The major disadvantage of this approach is the poor efficiency of utilization of the GlcNAz by the cells. All of the enzymes in the cellular machinery use their native substrates much more efficiently. In addition, high levels of endogenous UDP-GlcNAc that is present in

most cells effectively competes for utilization of the UDP-GlcNAz. Further, the sensitivity is exacerbated by the usual low stoichiometry of O-GlcNAcylation combined with the relative inefficiency of incorporation of the azido sugar. Unfortunately, lowering endogenous UDP-GlcNAc levels to increase efficiency of GlcNAz incorportaion by any of several means will generally also perturb the biological process of interest. Nonetheless, this approach is useful for studying O-GlcNAcylation on abundant proteins and perhaps on those where the saccharide is most rapidly cycling.

β-ELIMINATION FOLLOWED BY MICHAEL ADDITION (BEMAD/MAP) BASED ENRICHMENT

The O-b-GlcNAc glycosidic linkage is much more alkali labile than O-a-GalNAc or O-phosphate. Since O-GlcNAc is very sensitive to b-elimination by alkali, and is very labile in the gas phase upon CID fragmentation, early site mapping studies used mild b-elimination of O-GlcNAc peptides and the resulting loss of water at the Ser(Thr) attachment site (18 m/z loss) to map sites [70]. Later, this method was improved by using Michael addition chemistry to attach dithiothreitol (DTT) to the b-eliminated O-GlcNAc sites (m/z increase of 136). This method was called BEMAD [65]. BEMAD provided for several advantages: 1) The attached DTT moiety is stable to standard MS/MS conditions, such as CID, and modified peptides fragment normally. 2) DTT allows for affinity enrichment of modified peptides using thiol-affinity chromatography. 3) Deuterium containing DTT is readily available allowing for comparative semi-quantitative mass spectrometry [65,71,72]. The biggest disadvantage of the BEMAD method for site mapping is that it is indirect. In theory, alkali-induced elimination of any moiety at a Ser(Thr) residue could result in a false positive assignment as an O-

GlcNAc site. Therefore, the use of BEMAD for site mapping requires many careful controls [71,73,74], and confirmation by independent methods.

CHEMOENZYMATIC ENRICHMENT OF O-GLCNAC PEPTIDES

Most recently, chemoenzymatic approaches have been used to tag and enrich O-GlcNAcylated peptides. A commercially available kit (Click It kit, Invitrogen) has simplified the use of this approach. A genetically engineered (Y289L muation) galactosylransferase that has a larger donor-substrate binding pocket, is used. The mutant GalT can efficiently transfer N-azidoacetylgalactosamine (GalNAz) to the O-GlcNAc moiety [75]. Biotin is then quantitatively attached to GalNAz via click chemistry using a copper catalyst [76]. The labeled protein /peptide can then be enriched through avidin/streptavidin affinity chromatography. Even though this tagging and enrichment method greatly enriches the O-GlcNAcylated proteins/peptides, the enriched sample is still not suitable for direct MS/MS analysis. The tag mass attached to the O-GlcNAc is too big, which results in even more severe ion suppression compared with nonenriched O-GlcNAcylated peptide. The biotin moiety fragments excessively during MS/MS complicating intepretation of the spectra and the saccharide moiety bearing the GalNAz-biotin is still very labile during MS/MS fragmentation, preventing direct site mapping.

The combined use of BEMAD and the chemoenzymatic enrichment does allow for direct site mapping and the high specificity of the enrichment gives a much higher confidence in the validity of sites idenfified. The combined approaches have been used to map O-GlcNAc sites on on vimentin and Calcium/Calmodulin-dependent kinase IV (CaMKIV) [64,66]. A complex mixture of human erythrocyte proteins was also analyzed

using this the chemoenzymatic enrichment followed by BEMAD, resulting in the identification of thirty five O-GlcNAc sites [72]. A similar enrichment method that instead uses an UDP-ketogalactose and hydrazide coupling of biotin also works well and has been used in cell lysates and brain tissue [77,78].

PHOTOCLEAVABLE BIOTIN (PC-BIOTIN) BASED ENRICHMENT OF O-GLCNAC PEPTIDES

A major problem with enriching O-GlcNAc using biotin/avidin based affinity methods is the difficulty of getting quantitative recovery of the bound glycopeptides. Thus, building upon Olejnik's report of photocleavable biotin reagent [79], a photocleavable biotin-alkyne reagent suitable for use in O-GlcNAc site mapping was developed [49]. The same chemoenzymatic attachment method (described above) is used, but the tagged glycopeptides are quantitatively released by a cleavage reaction catalyzed by ultraviolet light (365 nm). A major advantage of this method, in addition to its efficiency, is that the released peptides now carry a basic aminomethyltriazolyl acetylgalactosamine group, which results in a 3+ or higher charge state for the tryptic O-GlcNAc peptides. The charged moiety remaining after ultraviolet light cleavage not only facilitates electron transfer dissociation mass spectrometry, but also produces diagnostic fragment ions (m/z of 300.2 and 503.1) allowing detection of O-GlcNAc peptides in complex mixtures by standard CID MS [49].

ADVANCES IN MASS SPECTROMETRY THAT FACILITATE ANALYSES OF O-GLCNAC

The enrichment methods, described above, have solved the analysis problems associated with low stoichiometry and ion suppression by unmodified peptides. In addition, the sensitivity of modern mass spectrometers continues to increase dramatically.

COLLISION INDUCED (ACTIVATED) DISSOCIATION (CID/CAD)

O-GlcNAc attachment to Ser/Thr is labile. In CID/CAD, the energy required to break the O-GlcNAc linkage is much lower than that required to break peptide bond. CID/CAD almost always results in a neutral loss of the saccharide, which can indicate the existence of O-GlcNAclation on the peptide, but provides no site information [80-82]. In addition, due to the loss of kinetic energy associated with the neutral loss of the saccharide, CID/CAD is generally very poor at producing enough peptide fragmentation of the O-GlcNAc peptide for sequencing. However, in ion-trap mass spectrometers it is possible to perform an MS^3 CID/CAD and obtain quality peptide sequence, but only after the sugar is lost. In contrast, DTT attached by the BEMAD procedure, is not lost during CID/CAD and the DTT-peptides fragment as well as unmodified peptides. A combination of BEMAD and CID/CAD, has been used to map sites on synapsin I, nuclear pore complex proteins, actin, myosin, and IRS-1 [83,84].

ELECTRON CAPTURE DISSOCIATION (ECD) AND ELECTRON TRANSFER DISSOCIATION (ETD) MASS SPECTROMETRY

Electron capture dissociation (ECD) uses low energy electrons to react with peptide cations in the magnetic field of a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer [85]. Unlike CID/CAD mass spectrometry, the O-GlcNAc linkage is not broken by ECD of peptide ions [67]. However, the high cost and high level of expertise required to operate and maintain FT-ICR mass spectrometers limits their widespread use. Recently, an ionization method similar to ECD, called electron transfer dissociation (ETD) has been developed which works well in lower cost ion trap mass spectrometers [86,87]. During ETD an electron is transferred in the gas phase from a radical anion to a protonated peptide to induce cleavage of the Cα-N bond. Like CAD, ETD preserves the O-GlcNAc and other peptide modifications, while efficiently fragmenting the peptide. The development of ETD is a breakthrough in the detection and analysis of O-GlcNAcylation. ETD mass spectrometry has recently been used to map O-GlcNAc on paxillin, a focal adhesion adaptor protein, IRS-1 and Forkhead box O1 (FoxO1), and PPARγ coactivator-1 (PGC-1α) [14,63,88,89]. By combining ETD with sWGA chromatography enrichment, greater-than fifty previously unknown O-GlcNAc sites on murine postsynaptic density proteins were mapped [90]. Chemoenzymatic enrichment using a photocleavable biotin tag, combined with ETD mass spectrometry, enabled the mapping of over one hundred and forty O-GlcNAc sites on proteins involved in the regulation of cell division [91].

QUANTITIATIVE ANALYSIS OF O-GLCNAC SITE OCCUPANCY

STABLE ISOTOPE LABELING WITH AMINO ACIDES IN CELL CULTURE (SILAC)

In order to quantitatively compare O-GlcNAc levels on different proteins or at different sites, stable isotope labeling with amino acids in culture (SILAC) [92,93] when combined with the mass spectrometric methods discussed above, has proven to be a method of choice. SILAC was used to test quantitative O-GlcNAcylation changes in COS7 cells after only a single kinase, glycogen synthase kinse-3 (GSK-3), was inhibited [91]. Major disadvantages of SILAC include the high cost of isotope containing amino acids, and that the metabolic incorporation of the labeled amino acids requires living samples, either in culture or at the whole organism level.

IN VITRO METHODS FOR ISOTOPICALLY LABELING O-GLCNAC PEPTIDES

Recently, two similar *in vitro* methods have been used for quantitative analysis of O-GlcNAcylation by isotopically labeling peptides with different tags [72,94]. One approach is "Tandem Mass Tags"(TMT) and the other is "Isobaric Tags for Relative and Absolute Quantification"(iTRAQ) [95,96]. Peptides are labeled with different tags, which upon tandem mass spectrometry are cleaved into different m/z reporter ions. Each tag introduces a unique mass shift for the labeled peptides. The advantage of these tags is that they allow for multiplexing, and peptides obtained from tissues or other sources can be efficiently labeled *in vitro*. A similar peptide labeling method makes use of reductive amination to incorporate stable isotopes into N-terminal amines and ε-amino groups of lysine residues [97]. Using this approach, a quantitative isotopic and chemoenzymatic tagging (QUIC-tag) method was developed for O-GlcNAc analysis. In this method, O-GlcNAcylated peptides are first enriched by the modified chemoenzymatic enrichment method, discussed above, and then the biotin labeled O-GlcNAcylated peptides are dimethylly isotopically labeled. By using the

QUIC-tag approach, many quantitative O-GlcNAc changes on proteins after inhibition of O-GlcNAcase, as well as after different stimulations of the brain were identified [98].

β-ELIMINATION MICHAEL ADDITION WITH DENTISTY TAGGED DITHIOTHRITOL (DTT)

Using chemoenzymatic enrichment (as above) combined with BEMAD, but using deuterated DTT (DTT-$^{3}D_{6}$) for one sample and light DTT for the other samples, allows for direct comparison of O-GlcNAc site occupancy, as long as the necessary controls are performed. For example, this approach was used with good results to compare O-GlcNAc site occupancy between sites on proteins from erythrocytes obtained from either normal of normal and diabetic patients [72]. The advantage of this approach is that it can be performed on inexpensive ion trap mass spectrometers using CID/CAD tandem mass spectrometry. The disadvantage (as discussed above) is that detection of O-GlcNAc is indirect.

With the development of these new methods, the pace of identification and site mapping of O-GlcNAcylated proteins is rapidly increasing. In fact, the total O-GlcNAc sites mapped within the past two years exceeds that known during the past twenty-years [1]. Figure 2 summarizes the current approaches available for identification and site-mapping O-GlcNAc on proteins.

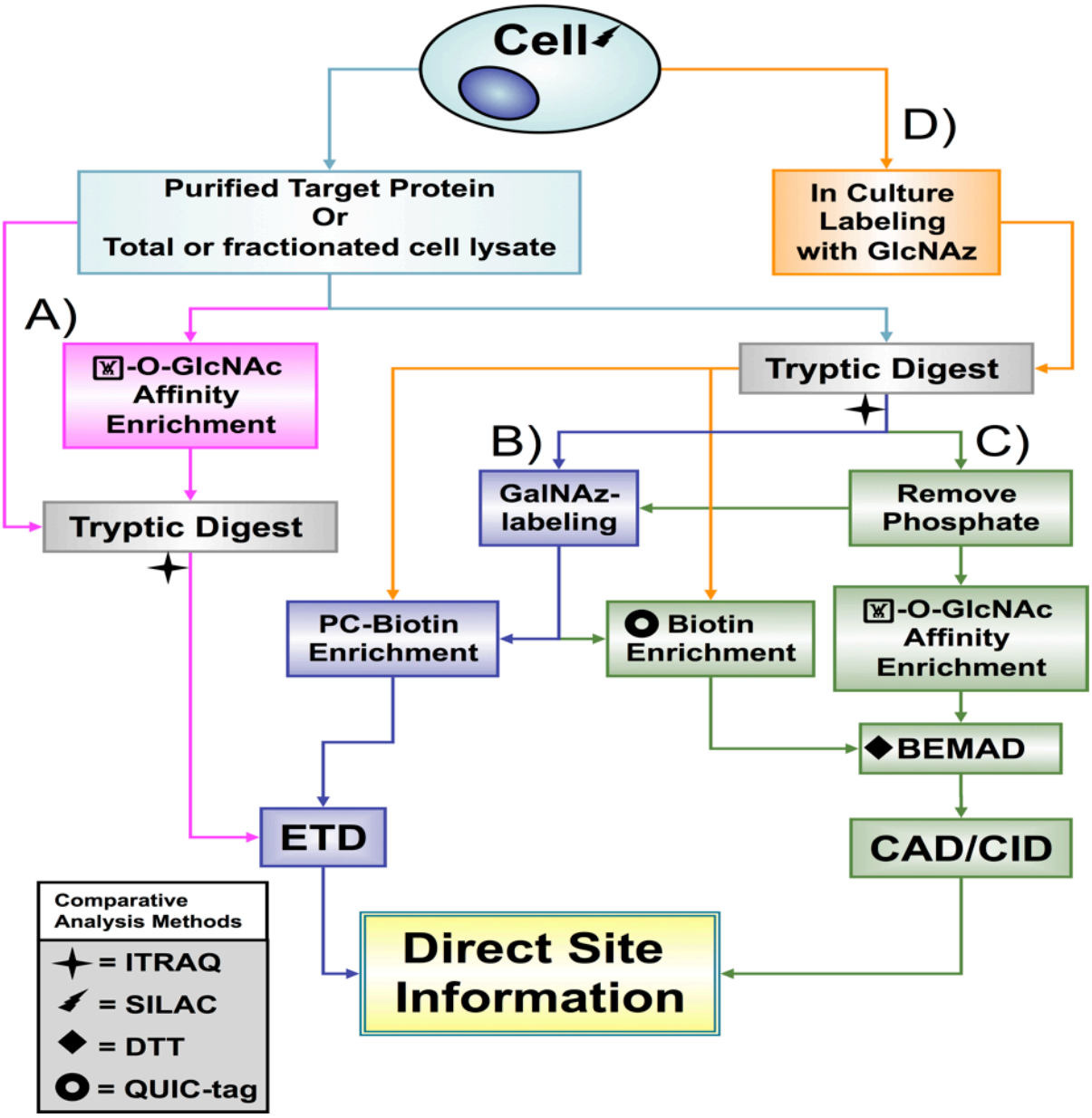

Figure 2. O-GlcNAc enrichment and site mapping methods. O-GlcNAc sites can be mapped by: A) directly analyzing the purified target protein, cell extracts, fractionated lysates, or samples enriched for O-GlcNAc by immune affinity chromatography by ETD MS. B) Enriching for O-GlcNAc modified peptides, derived from the purified target protein, total cell lysates or fractionated lysates, by the PC-biotin method and subsequently analyzing by ETD MS. C) Modifying O-GlcNAc sites of enriched O-GlcNAc modified peptides using BEMAD and analyzing by CAD/CID MS. D) In culture labeling the proteins with GlcNAz and processing through PC-biotin or biotin enrichment methods. Various quantitative peptide tags may be introduced at specified steps to allow for comparative analysis of multiple sets of samples.

EXTENSIVE CROSSTALK EXISTS BETWEEN PHOSPHORYLATION AND O-GLCNACYLATION

O-GlcNAcylation occurs on Ser(Thr) side chains of proteins at sites that may also be phosphorylated. Thus far, virtually every O-GlcNAcylated protein can also be phosphorylated [99]. After several O-GlcNAc sites were mapped and discovered to also be phosphorylation sites a "Yin-Yang" model was proposed for the relationship between O-GlcNAcylation and phosphorylation [100]. This model suggested that O-GlcNAcylation is reciprocal to phosphorylation with mutually exclusive occupancy at the same site on a polypeptide. While largely correct for many proteins, the Yin-Yang model is an over-simplification. Now that hundreds of O-GlcNAc sites have been mapped, and some cellular stimuli increase both modifications, it is clear that the interplay between O-GlcNAcylation and phosphorylation is both complex and very extensive.

GLOBAL INTERACTIONS BETWEEN O-GLCNACYLATION AND PHOSPHORYLATION

A competitive or reciprocal relationship between O-GlcNAcylation and phosphorylation has indeed been found in several global analyses. Global O-GlcNAc levels change after using various kinase activators and inhibitors. For example, activation of protein kinase A or protein kinase C decreased global O-GlcNAcylation and inhibitors of these kinases increased global O-GlcNAcylation [101]. Okadaic acid (OA), a broad spectrum phosphatase inhibitor has been used to explore the global interplay between O-GlcNAcylation and phosphorylation. Indeed, in some cells, OA short-term treatment decreases O-GlcNAcylation [102]. However, since OA also induces a stress response that

increases O-GlcNAcylation of many proteins, these types of global treatments are not very informative.

In hippocampal neuronal synapses, pharmacological elevation of O-GlcNAc *in vivo*, concomitantly increases phosphorylation of Synapsin I/II at serine^{9}(PKA site), serine$^{62/67}$(Erk1/2 site), serine^{603}(CaMK II site)[103]. These and other studies indicate that the nature and direction of the crosstalk between these two post-translational modifications depends upon not only the protein but also the specific modification site.

Chemoenzymatic enrichment methods and advances in mass spectrometry have allowed global comparisons of O-GlcNAcylation and phosphorylation at the individual site level. In studies to determine the affects of inhibiting glycogen synthase kinase 3 (GSK3) on O-GlcNAcylation, of the forty-five identified O-GlcNAcylated proteins, at least ten proteins (mostly cytoskeletal proteins) had increased O-GlcNAcylation, while nineteen other proteins (mostly RNA binding proteins) had decreased O-GlcNAcylation [66]. A recent study examined the affects of globally increasing O-GlcNAcylation, by inhibiting O-GlcNAcase, on the quantitative site occupancy of phosphate at over seven hundred phosphorylation sites in non-stimulated NIH3T3 cells increased O-GlcNAcylation quantitative site occupancy at over seven hundred phosphorylation sites [104]. Forty-eight percent of the phospho-sites were not actively cycling in these non-stimulated cells, as judged by insensitivity to okadaic acid (OA). However, of the remaining actively cycling phospho-sites nearly all had their level of phosphorylation affected by altered O-GlcNAcylation. Elevated O-GlcNAcylation significantly reduced phosphorylation at about two hundred and eighty sites and significantly increased phosphorylation at one hundred and forty-nine sites. These studies revealed the surprisingly extensive level of crosstalk between these two most abundant post-translational

modifications. This extensive crosstalk not only results from steric competition at the same or proximal attachment sites, but also it is now clear that phosphorylation regulates both OGT and OGA, and O-GlcNAc is being found to regulate an increasing number of kinases [64,105].

Overexpression of OGT causes defective cytokinesis increasing polyploidy in cells, a feature common to many cancer cells [106]. In order to understand the molecular basis for OGT's induction of defective cell division, a glyco-, phospho-SILAC proteomic analysis, using chemoenzymatic enrichment with PC-biotin and a combination of CID and ETD mass spectrometry was carried out on spindle/midbody preparations isolated from HeLa cells overexpressing either GFP (control) or OGT (experimental) [91]. A comparable number of phosphorylation and O-GlcNAcylation sites were mapped (three hundred and fifty phosphorylation sites on one hundred and ninety proteins vs one hundred and forty-one O-GlcNAcylated peptides on sixty-four proteins). Seventeen percent of the phosphosites showed more than a fifty-percent decrease upon a two-fold overexpression of OGT and the resulting increase in global O-GlcNAcylation. Some (seven percent) phosphorylation sites had a more than two-hundred percent increase in site occupancy. These changes resulted in pronounced inhibition of the cyclin dependent kinase I (CDK 1) signaling pathway, and alterations in polo kinase and aurora kinase signaling, partially explaining the cytokinesis defects induced by OGT over-expression. These data clearly exemplify the importance of the interplay between O-GlcNAcylation and phosphorylation in cellular signaling pathways.

Hyperphosphorylation of the microtubule bundling protein, tau is a characteristic of of Alzheimer's disease (AD). It has also been known for sometime that tau is heavily O-GlcNAcylated in normal brain [107]. These findings led to the proposal that defective

glucose metabolism in the brain results in lower O-GlcNAcylation of brain proteins, including tau, leading to abnormally increased hyperphosphorylation associated with AD [108].When tau becomes hyperphosphorylated, it forms visible tangles within neurons and is referred to as paired helical filamentous tau (PHF-tau). To date, more than thirty phosphorylation sites have been mapped on PHF-tau, and many of them have been used to produce site-specific phosphorylation antibodies [109]. While there are more than twelve putative O-GlcNAc sites on tau, only a few sites have been mapped and no site specific antibodies have been made [107]. Increased O-GlcNAcylation negatively regulates tau phosphorylation at multiple sites, and hyperphosphorylated tau has decreased O-GlcNAcylation. Several studies have shown that decreased flux through the HBP or decreased expression of OGT result in tau hyperphosphorylation in animal models [110,111]. The potent OGA inhibitor, Thiamet G (TMG), increases global O-GlcNAcylation effectively in whole animals and crosses the blood-brain barrier [112]. TMG treatment increased O-GlcNAcylation and decreased phosphorylation at pathologically relevant sites on tau, Thr^{231} and Ser^{396} in PC12 cells and Ser^{422} in rat cortex and hippocampus [112].

Endothelial nitric oxide synthase (eNOS) is regulated by reciprocal O-GlcNAcylation/phosphorylation. Ser^{1177} phosphorylation of eNOS by AKT kinase activates the enzyme and is important in positively regulating penile erection, and phosphorylation on Thr^{495} of eNOS acts as a negative regulator. Ser^{615} and Ser^{633} phosphorylation can also acivate eNOS. In diabetic rats excessive O-GlcNAcylation at Ser^{1177} prevents AKT activation of eNOS without affecting phosphorylation at Thr^{495}, Ser^{615} and Ser^{633} [113], and in humans with diabetes O-GlcNAcylation of eNOS contributes to erectile dysfunction [113].

Upon activation of the insulin signaling cascade, phosphorylation of AKT at Thr^{308} is increased. Concomitantly, phosphorylations on IRS-1 at Ser^{307} and $Ser^{632/635}$ are decreased. When OGT is over-expressed in mouse liver, increased O-GlcNAcylation of both AKT and IRS-1 occurs, but results in decreased phosphorylation at Thr^{308} of AKT and increased phosphorylation at Ser^{307} and $Serine^{632/635}$ on IRS1. These findings support the hypothesis that the crosstalk between O-GlcNAcylation and phosphorylatioin is complex and is not simply a 'yin-yang' relationship. The two modifications work synergistically to regulate insulin signaling [114].

The cycling dynamics of O-GlcNAcylation and phosphorylation are also differentially regulated. For example, on the transcription factor, Sp1, O-GlcNAcylation of Sp1 peaks at thirty minutes post insulin stimulation, followed by a steady decline, and reaches low levels by four hours. In contrast, the insulin stimulated phosphorylation on Sp1 increases gradually and reaching a peak around four hours after insulin treatment [115] [116].

Despite many exceptions, there are numerous proteins, such as c-Myc, estrogen receptor β (ER-β) and Akt [105], in which the O-GlcNAc moiety and the phosphate residue compete for the same hydroxyl group, as proposed in the 'yin-yang' model. One of the best-studied examples is the c-Myc oncogene protein. The protooncogene, *c-myc*, regulates transcription of genes involved in cell proliferation, apoptosis and metabolism [117]. The major O-GlcNAcylation site on c-Myc was mapped to Thr^{58}, which is also a GSK3β phosphorylation site within the transaction domain [60]. Frequent mutation of this site is found in human lymphomas, demonstrating the importance of this site in tumor progression [117]. Antibodies specifically detecting O-GlcNAcylated Thr^{58}, phosphorylated Thr^{58}, as well as Thr^{58}-unmodified c-Myc were used to study the respective roles of each modification.

Serum starvation stops cell growth and greatly increased Thr^{58} O-GlcNAcylation. In contrast, when cells were stimulated to grow by serum addition, O-GlcNAcylation at Thr^{58} very rapidly disappears and is replaced by increased Thr^{58} phosphorylation by GSK3b. The proximal site, Ser^{62}, is a priming phosphorylation site and a substrate for mitogen activated protein kinases. Phosphorylation at Ser^{62} is necessary for Thr^{58} phosphorylation by GSK3β. When Ser^{62} is mutated to alanine, not only does Thr^{58} no longer become phosphorylated, but also the extent of O-GlcNAcylation at Thr^{58} increases dramatically. Lithium chloride, a GSK3β inhibitor, also increased O-GlcNAcylation at Thr^{58} similar to the Ser^{62}Ala mutation [118]. Estrogen receptor b (ER-β) is reciprocally O-GlcNAcylated/phosphorylated at Ser^{16} [55,119]. Ser^{16} is within a region of ER-b with high Pro, Glu, Ser and Thr (PEST) score, which is related to rapid protein degradation. Site-directed mutagenesis comparisons between wild type ER-b to mutants, $S^{16}A$, $S^{16}E$ suggested that the O-GlcNAcylated ER-b was much more stable, but less transcriptionally active, while the phosphorylated ER-b was transcriptionally more active, but rapidly degraded [120]. Consistent with the biological studies, NMR analysis and molecular dynamics simulation of synthesized peptides, suggested that phosphorylation in Ser^{16} discourages b-turn formation at the N-terminus of the protein, resulting in a more extended structure, while Ser^{16} O-GlcNAcylated peptides promoted b-turn formation, adopting a more stable structure, which to some degree might explain why O-GlcNAcylation on Ser^{16} can stabilize the protein [121]. A similar dynamic interplay between O-GlcNAcylation and phosphorylation occurs on the C-terminal domain (CTD) of RNAP II. The CTD of RNAP II is extensively phosphorylated during the transition from the preinitation complex to the assembly of the elongation complex during transcription. On a subset of the RNAPII, the CTD contains no phosphate, but is extensively

O-GlcNAcylated [122]. The mutually exclusivity of the two modifications on CTD was shown *in vitro*. OGT was completely unable to O-GlcNAcylated a CTD substrate that contained even a single phosphate moiety. Likewise, the CTD kinase was unable to phosphorylate a CTD containing only a single O-GlcNAc, despite many other sites being available [123]. Another example of direct reciprocity is that of Iκ kinase β (IKKβ). IKKb is O-GlcNAcylated at Ser^{733} in its C-terminus, which is also an inactivating phosphorylation site. The reciprocal interaction in this site is responsible for regulating IKKβ catalytic activity [124].

Perhaps more common than direct site competition is the interplay that occurs between O-GlcNAcylation and phosphorylation at proximal sites. It should be noted that the Stoke's radius of an O-GlcNAc is several times larger than that of a phosphate moiety, despite the lack of a charge on the sugar (Figure 3). The important tumor suppressor, p53, bears proximal O-GlcNAc (Ser^{149}) and phosphate (Thr^{155}) residues. O-GlcNAcylation of Ser^{149} reduces phosphorylation at Thr^{155}, which subsequently blocks the interaction between Mdm2, an E3 ubiquitin lyase, and p53, a prerequisite for proteasomal degradation of p53. Thus, increased O-GlcNAcylation of p53 at Ser^{149} results in decreased p53 ubiquitination and stabilizes the protein p53 [125]. Mutation of the Ser^{149} O-GlcNAc site on p53 to alanine increases phosphorylation at Thr^{155}. Three dimensional structural analysis shows that these two sites are located on a flexible loop with close proximity of only 11.58 Å, suggesting that there might not be enough space for dual occupancy of both phosphate and O-GlcNAc groups at these two sites.

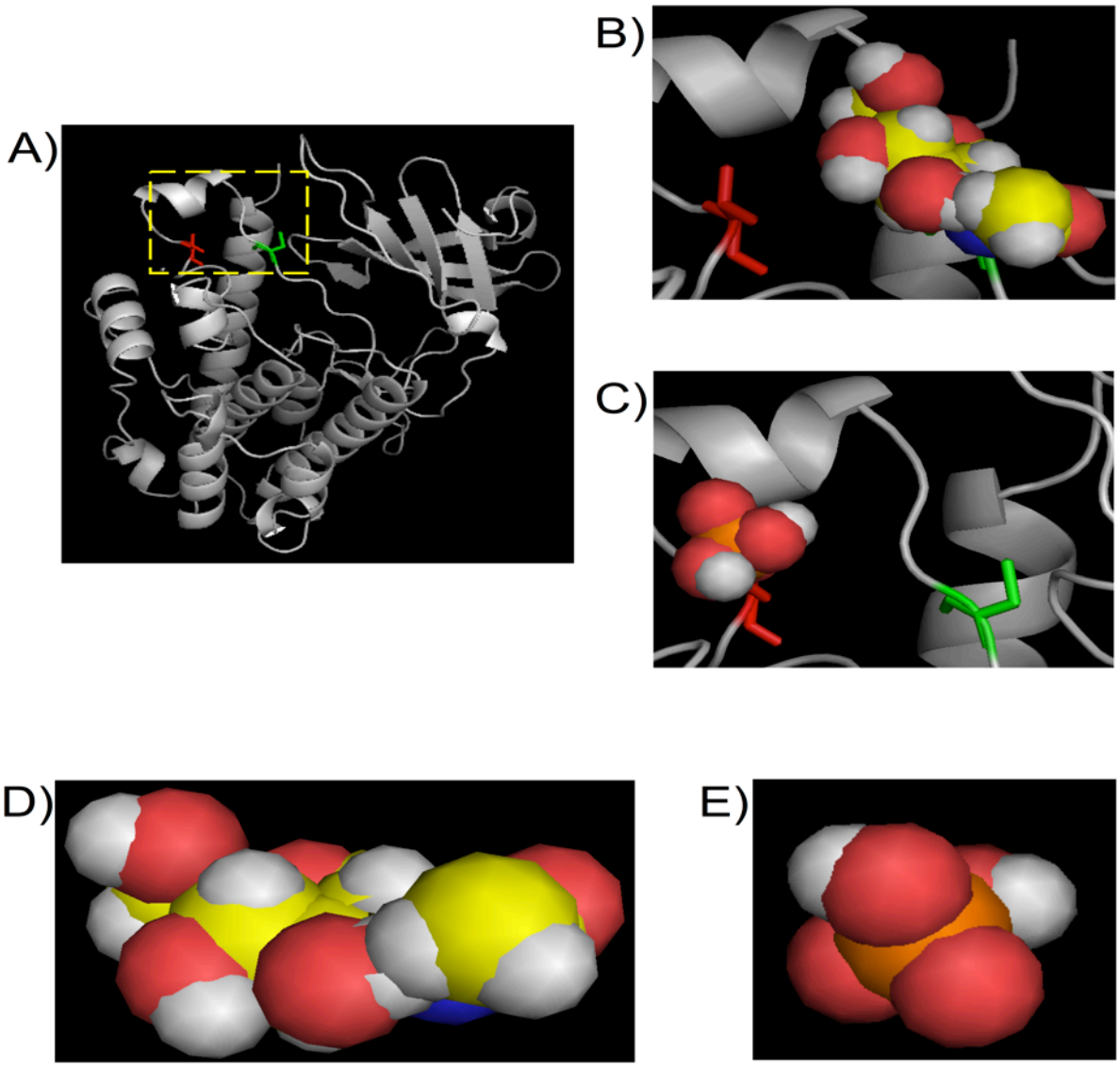

Figure 3. O-GlcNAc vs. O-phosphate modification of CaMKIV. The structure of CaMKIV was obtained through the automated comparative modeling program SWISS-MODEL. Mouse CaMKIV sequence (PO8414) was analysed by the automated mode. The X-ray crystal structure of Human CaMKIV (PDB code 2w4oA) was chosen as a closet match template to create a model of CaMKIV. The PDB created through the homology-based modeling was then analysed using MacPymol to create the figure presented above. A) Unmodified CaMKIV model, the protein is depicted to show the secondary structure (grey) with Ser 189 (green) and Thr200 (red) represented in stick model. The boxed area was enlarged to show the modified forms. B) O-GlcNAcylated CaMKIV model with O-GlcNAc (carbon is yellow, hydrogen is white, nitrogen is blue, oxygen is pink, sphere model) attached to Ser189. C) O-phosphate CaMKIV model with phosphate (phosphate is orange, others as in B.) attached to Thr200. D) Space filling model of a GlcNAc residue E) Space filling model of a phosphate residue.

Site mapping studies have recently revealed a similar proximal interplay between O-GlcNAcylation and phosphorylation on CaMKIV, a key kinase regulating gene expression in neurons and other cells. CaMKIV is predominantly expressed in brain, T-lymphocytes and in the testis. It is a multifunctional Ca2+/calmodulin dependent Ser/Thr kinase, that regulates downstream protein activities according to changes in intracellular Ca2+ concentrations. CaMKIV is O-GlcNAcylated and phosphorylated on several sites. Five O-GlcNAcylation sites were mapped on CaMKIV and O-GlcNAc on Ser189, Thr57/Ser58 were evaluated for their interactions with phosphorylation, mainly at the key regulatory phosphorylation site at Thr200. Phosphorylation at Thr200, which is the target of CaMK kinase (CaMKK), increases CaMKIV kinase enzymatic activity. Upon neuronal depolarization using ionomycin to increase intracellular Ca2+, phosphorylation at Thr200 rapidly increases peaking at two minutes, and decreasing after five minutes. In contrast, O-GlcNAcylation at Ser189 reciprocally decreased at 2 min, and returned to basal level at around 10min. Mutating Ser189 to alanine dramatically increases both the phosphorylation of CAMKIV at Thr200 and its constitutive enzymatic activity. Mutation of Thr57/Thr58 to alanine inactivates the kinase. Analysis of the three-dimensional structure of CAMKIV revealed that all three of these O-GlcNAc sites are located within the catalytic cleft, near the ATP binding residues Lys75 and Asp185 [64].

As discussed above, O-GlcNAcylation and phosphorylation certainly have extensive crosstalk due to steric competition of the modifications themselves at the same or proximal sites on proteins. However, they also influence each other by each modification regulating the activities or localizations of the other's cycling enzymes. For example, OGT is directly activated by tyrosine phosphorylation and is itself O-GlcNAcylated [15], and CAMKIV is

directly inhibited by O-GlcNAcylation [64]. Src, a well known tyrosine kinase important to cancer, phosphorylates OGT *in vitro* [126]. Upon insulin stimulation, OGT is recruited to the plasma membrane by binding phosphatidylinositol 3,4,5-triphosphate (PIP3). Insulin receptor tyrosine phosphorylates OGT, increasing its activity [114,126]. OGT also forms a stable and active complex with Ser/Thr protein phosphatase PP1β and PP1γ in rat brain [127]. Additionally, PP1 and the mitotic kinase Aurora B form a complex with OGT and OGA during M phase of the cell cycle. The Aurora B kinase inhibitor, ZM447439 (ZM), disrupts Aurora B's localization to the mid-body during cytokinesis, as well as its staining co-localization with OGT. The transient complex that forms at the midbody during cytokinesis regulates both the phosphorylation and O-GlcNAcylation of many proteins that regulate cell division, including vimentin [6].

In summary, phosphorylation and O-GlcNAcylation can reciprocally or synergistically interact with each other on the same sites or proximal sites within proteins (Figure 4). This interplay serves to fine tune signaling, transcription and other cellular processes in response to nutrients and stress. This dynamic interplay is further complicated by the regulation of OGT and OGA by phosphorylation and the increasing numbers of kinases that appear to be regulated by O-GlcNAcylation.

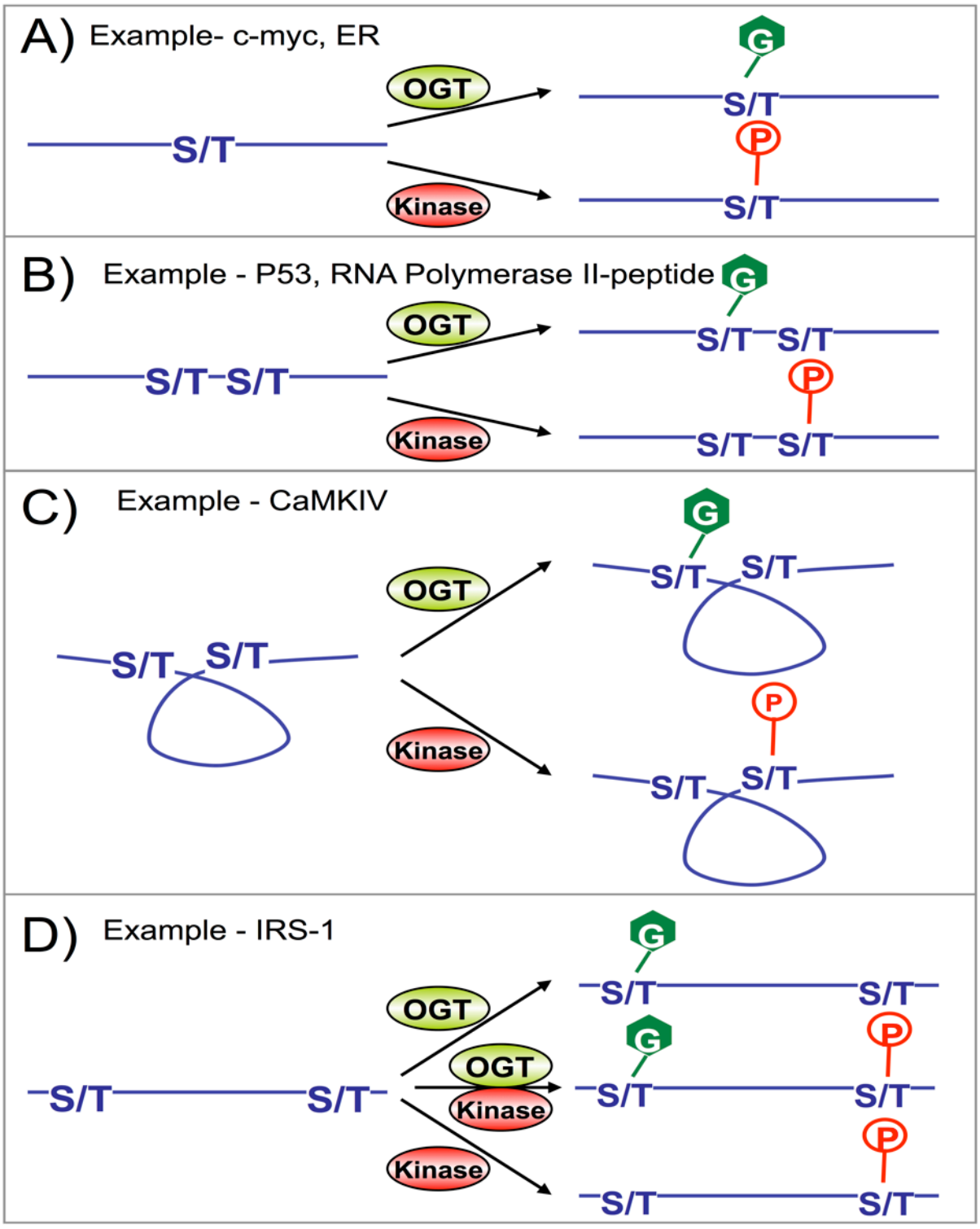

Figure 4. Interplay between O-GlcNAcylation and phosphorylation. A) Single site reciprocal interplay B) Proximal sites reciprocal interplay C) Spatially proximal sites with reciprocal interplay D) Distant sites with varied interplay

HOW DOES A SINGLE CATALYTIC PEPTIDE (OGT) SPECIFICALLY MODIFY MORE THAN A THOUSAND DIFFERENT PROTEINS?

As mentioned above, there are three hundred and eighty-five known Ser/Thr kinases in humans, each kinase functions in its own specific substrates and has its own regulation signal[128]. However, in mammals there is only one known gene for OGT and only two known in plants. Thus, the site specificity of phosphorylation and O-GlcNAcylation are determined by very different mechanisms. How does this single protein specifically modify so many different sequences within proteins? There is precedence for this problem in biology. For example, there is only a single gene encoding RNAP II's catalytic subunit. How then does RNAP II specifically transcribe in a regulated way from so many different promoters? It is well documented that RNAP II's activity is determined by its forming many transient protein complexes that form holoenzymes with precise specificity. Current data, suggests that this mechanism is how OGT's specificity is also determined. That is, in a living cell many different holoenzymes, that are comprised of OGT and other polypeptides, form under specific conditions and the many resulting OGT complexes serve to specifically target the catalytic subunit to its target proteins (Figure 5). In addition, OGT also has specific sequence requirements, but not an absolute consensus sequence.

Early work showed that adapter or targeting proteins for OGT bind to its TPR-repeats to target the enzyme to transcription complexes and other targets [129-131]. Many transcription factors, such as Sp1, c-myc, are O-GlcNAcylated [60,132]. O-GlcNAcylation of

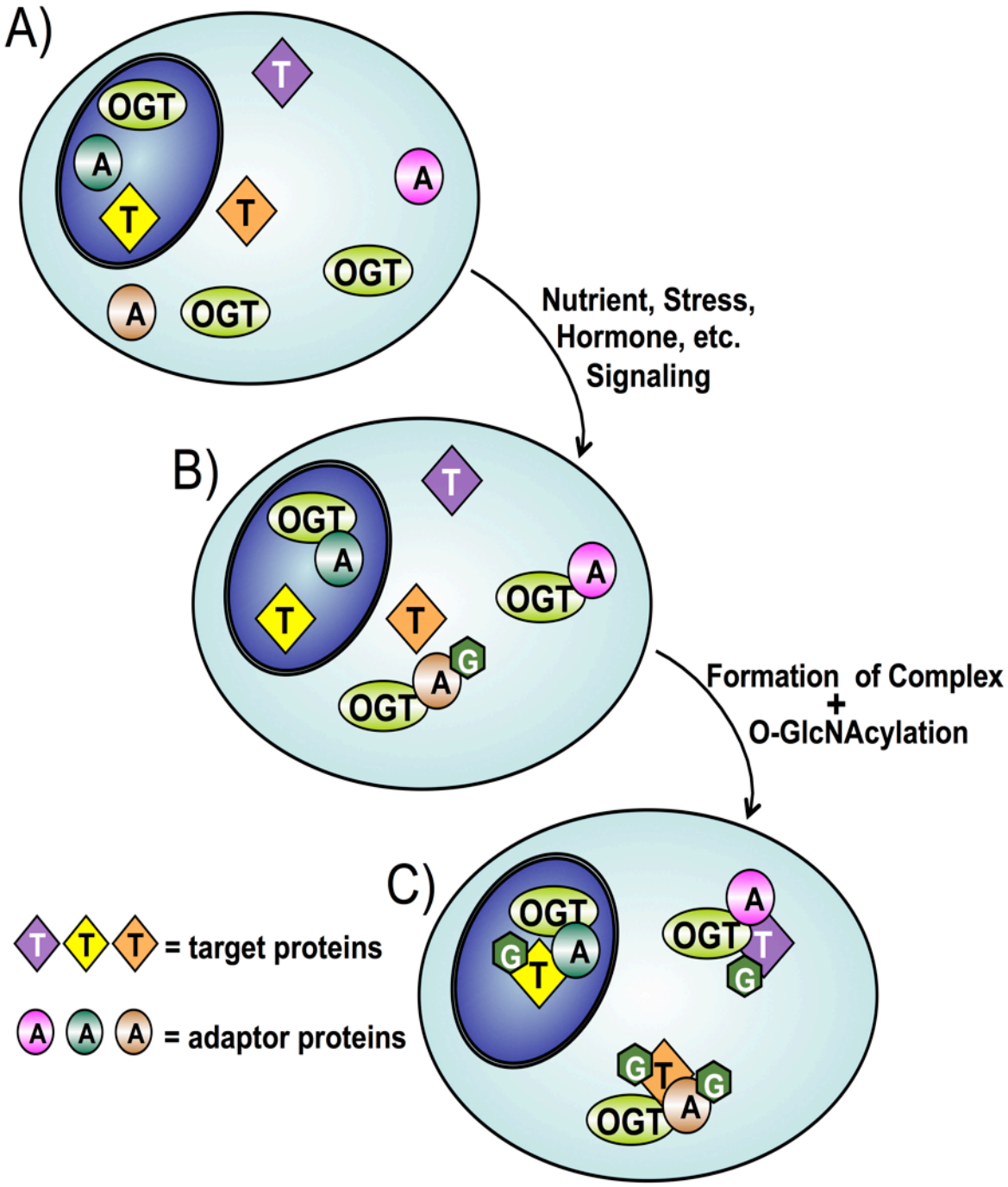

Figure 5. OGT targeting to specific proteins by adaptor/targeting proteins. A) In the basal state OGT does not interact with an adaptor or target protein. B) Upon stimulation (such as nutrient, stress and hormone) OGT interacts with adaptor/targeting proteins. C) The adaptor/targeting protein recruits OGT to its target protein specifically increasing its rate of O-GlcNAcylation in signal dependent manner.

Sp1 inhibits its binding ability with other Sp1 proteins as well as TATA-binding protein-associated factor II 110 ($TAF_{II}110$) through breaking the hydrophobic interactions between theses proteins [132,133]. Homomultimerization of Sp1 is required to induce transcription activation, and Interaction with $TAF_{II}110$, is needed for binding with general transcription factor II D, and conducting DNA polymerase II dependent transcription [134].

The role of OGT in transcription repression was later clarified. By tethering OGT to the Gal4 DNA binding domain, it was shown recruitment of OGT to gene promoters enhanced the inhibitory effect in both basal and Sp1-activated transcription level. Later analysis showed OGT could be recruited to promoters by another transcription repressor, mSin3A, where histone deacetylases 1(HDACs) interacted with the complex, resulting in transcription repression. Truncation experiments indicated that the first 6 TPRs of OGT, which mediates protein-protein interaction, are the main motif interacting with the putative paired amphipathic helix (PAH) domains of mSin3A [9,129].

Through yeast two hybrid screenings, more and more adaptor proteins for OGT have been identified. And they were initially termed as OGT interacting protein (OIP). Firstly, two highly homologous proteins were isolated, $GABA_A$ receptor-associated protein (GRIF-1) and OIP106 (now it is known as Trak1). The interaction between OGT and either of these two proteins were confirmed both *in vivo and in vitro*. Consistent with previous results, it's the first 6 TPR domain of OGT that interact with these two adaptor proteins [131]. Confocal and electron microscopy data showed OIP106 colocalized with RNAP II, and the complex of OIP106, RNAP II and OGT was detected in Hela nuclear extracts. This raised the possibility that OIP106 and GRIF-1 may serve as targeting OGT to RNAP II and $GABA_A$ separately [130].

A following up large-scale yeast two-hybrid screen performed using human fetal brain cDNA library, identified 27 putative OGT binding proteins. These proteins belong to a wide range of functional classes, such as cytoskeletal proteins, transcription factors and proteins involved in development, metabolism. Of these 27 proteins, myosin phosphatase targeting subunit 1 (MYPT1), a known targeting regulatory subunit of PP1β, and coactivator-associated argininemethyltransferase 1 (CARM1) were confirmed to interact with OGT directly and affect OGT substrate specificity *in vitro*. The MYPT1 or CARM1 interacting proteins were enriched through recombinant protein conjugated column, and then sent for *in vitro* OGT labeling. When recombinant MYPT1 or CARM1 was added in the reaction system, OGT activity towards those MYPT1, CARM1 interacting proteins is increased. Interestingly, the O-GlcNAc of MYPT1 and OGT are also enhanced, suggesting that the adaptor proteins for O-GlcNAcylating MYPT1 are also within the enriched protein pool. *In vivo* knock down of MYPT1 by RNAi decreased O-GlcNAcylation of many proteins even though the activity of OGT and OGA were not changed. This experiment confirmed the existence of interaction between OGT and adaptor proteins. Changing the level of these adaptor proteins can alter OGT substrate specificity *in vitro* and global O-GlcNAcylation spectrum *in vivo* provided more evidence to support our hypothesis: OGT regulates its substrate specificity through forming holoenzyme with different adaptor proteins. Several other adaptor proteins have also been reported in recent years. Ataxin-10 interacts with OGT p110 in brain tissue, and this interaction was competed by the mitochondrial OGT form, OGT p78. *In vitro* OGT assay using PC12 cell extract shows Atx-10 can increase OGT activity by 2-fold [135].

This adaptor theory was also confirmed in the O-GlcNAcyaltion of several specific proteins [13,14]. For example, when Neuro-2a cells were derpived of glucose, OGT interacts with p38. Through a large-scale co-immunoprecipitation, NF-H was identified as a substrate for both p38 and OGT, thus a possible targeting protein candidate for OGT with adaptor protein p38 interaction. This was confirmed by pharmacologically inhibiting p38 activity, which abolished O-GlcNAcylation of NF-H induced by glucose deprivation even though the interaction of OGT with NF-H was not changed [13].

Another OGT-adaptor protein-substrate example comes from transcription factor FoxO and PGC-1α. The interaction among these three proteins was confirmed cultured rat hepatoma Fao cells. *In vitro* OGT assay showed PGC-1α increased OGT activity towards FoxO through increasing the interaction between OGT and FoxO. Glucose stimulation has been shown to enhance O-GlcNAcylation of FoxO and its transcriptional activity in Fao cell [89]. This increase of O-GlcNAcylation was also PGC-1α dependent.

These two examples showed not only the existence of OGT-adaptor proteins-target proteins interaction, but also how this interaction varies to extracellular changes, such as glucose deprivation and glucose introduction.

CONCLUSION

O-GlcNAcylation has been recognized playing an essential role in regulating many cellular processes, such as transcription, cell signaling, metabolism and protein stability (more for reviews [7,136]). Thus it's not surprising that O-GlcNAcylation is involved in various diseases, like cancer, diabetes as well as neurodegenerative disease and cardiovascular related protection (more for reviews [137,138]).

The development of various rapid, high efficient technologies for O-GlcNAc site mapping, enables us access many new truths about this common posttranslational modification, as well as improve current theory about the interaction between O-GlcNAcylation and phosphorylation. In the future, better understandings of how O-GlcNAcylation acts in regulating cell function, suvival and disease progression are available through taking these new technologies.

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CHAPTER TWO

THE CROSSTALK OF O-GLCNACYLATION AND PHOSPHORYLATION IN INSULIN SIGNALING

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ABSTRACT

Diabetes is an emerging global epidemic, of which, type II diabetes accounts for more than 90% of the cases. Development of insulin resistance, which shows dampened cellular response to insulin, heralds the onset of type II diabetes. Previous studies have identified many components involved in the insulin signaling cascade and revealed extensive regulation of insulin signaling by phosphorylation. Insulin Receptor Substrate 1(IRS1) is one of the most critical components in insulin signaling, whose phosphorylation is also key to regulating the insulin signaling pathway. O-GlcNAcylation, as phosphorylation, also occurs on Serine and Threonine residues, where it serves as a nutrient sensor and it often dynamically competes with phosphorylation. Recently O-GlcNAc modification has been detected on IRS1. My thesis project was to decipher the roles of O-GlcNAcylation in insulin resistance as well as to determine how O-GlcNAcylation on IRS1 regulates the insulin signaling cascade.

We first studied insulin signaling activity downstream of IRS1 in mouse adipocyte 3T3-L1 cells. We observed different insulin signaling activations under low and high cellular O-GlcNAcylation conditions. With low O-GlcNAcylation levels, when O-GlcNAc transferase is inhibited by AcSG treatment, insulin stimulation of IRS1/PI3K/AKT pathway was elevated, which is responsible for the metabolic actions of insulin, such as glucose uptake and gluconeogenesis. In contrast, when OGA is inhibited by Thiamet-G (TMG), resulting in high O-GlcNAcylation levels, the IRS1/RAS/ERK pathway controlling cell growth and differentiation is further activated by insulin. These observations indicated a role for O-GlcNAcylation in regulating the flux down divergent insulin signaling pathways

downstream of IRS1, and highlighted the importance of O-GlcNAcylation of IRS1. As we are interested in O-GlcNAcylation's role in insulin resistance, we later focused on the metabolic insulin signaling pathway, and confirmed that AcSG treatment not only boosted insulin stimulated glucose uptake, but it also enhanced the IRS1/PI3K/AKT signaling cascade. Inhibition of OGT also reversed insulin resistance caused by Tumor necrosis factor alpha (TNFα) pre-treatment. The laboratory is continuing to compare the overall phosphorylation changes on signaling molecules among high, normal and low O-GlcNAcylation levels.

Besides the overall insulin signaling changes, we examined phosphorylation changes on the IRS1 protein under various O-GlcNAcylation conditions. We first identified the phosphorylation pattern of IRS1 by two-dimensional electrophoresis. Most dramatic changes were observed on IRS1 under low O-GlcNAcylation condition, with IRS1 remaining in two isolated patterns: an increase in highly phosphorylated IRS1 and but also the presence of poorly phosphorylated IRS1. We further measured phosphorylation levels of IRS1 by phospho-specific antibody, which showed increased serine phosphorylation and decreased tyrosine phosphorylation on IRS1 with low cellular O-GlcNAcylation.

I also explored the site-specific function of O-GlcNAcylation on IRS1. Previous literature showed overexpressed IRS1 in a mammalian cell line is O-GlcNAcylated, we further showed O-GlcNAcylation also existed on endogenous IRS1. We consistently found elevated O-GlcNAcylated IRS1 in both type I and type II diabetes rat models, which indicated that O-GlcNAcylation of IRS1 was highly associated with diabetes progression. In order to clarify site-specific mechanisms of O-GlcNAcylation of IRS1 in type II diabetes, we mapped O-GlcNAcylation sites on IRS1 through combination of CAD-MS/MS and ETD-

MS/MS. Due to technology limitation, even though we know more sites exist, we only identified two novel O-GlcNAcylation sites, Ser635 and Ser1005 on mouse IRS1 protein, increasing the number of known O-GlcNAc sites on IRS1 to 8. Ser1005 O-GlcNAcylation on human and rat IRS1 have been shown to be involved in regulating PI3K recruitment to IRS1, and thus inactivating insulin signaling pathway. Through site-mutagenesis and protein engineering, we found O-GlcNAcylation of Ser635 inhibited overall O-GlcNAcylation on IRS1 by blocking its addition at other sites on the protein. Further study clarified a negative O-GlcNAcylation feedback existed between Ser635 and the C-terminal loci of IRS1 (including Ser1005 and other unmapped sites), thus the overall effect of O-GlcNAcylation on Ser635 is to inhibit addition of O-GlcNAc on the C-terminus of IRS1, which results in elevated recruitment of PI3K, which subsequently activates the IRS1/PI3K/AKT signaling pathway.

INTRODUCTION

Obesity/diabetes is a rising epidemic in the world. In which, type II diabetes accounts for 90-95% of all the diagnosed cases of diabetes. The vast majority type II diabetic patients are insulin resistant, which results in dampened signaling activation under insulin stimulation [1]. Under insulin stimulation, both Ras-ERK and PI3K-Akt signaling can be activated. These two signaling pathways might also intersect to regulate each other and co-regulate downstream functions [2]. Once insulin binds with the two extracellular α-subunits of insulin receptor, the two intracellular β-subunits transphosphorylate one another, further increase the tyrosine kinase activity. The insulin receptor tyrosine kinase continues to phosphorylate the downstream molecules. IRS and Shc are two of more than ten substrates identified in the insulin signaing cascades.

Phosphorylated tyrosines on IRS act as docking sites for PI3K, which in turn becomes activated. The activated PI3K contains two subunits: p110 calalytic subunit and p85 catalytic subunit. PI3K stimulates production of PI3-phosphate, which activates Akt through activating PDK1 and bringing Akt close to the PDK1. Akt has numerous and diverse targets, such as GSK3, FOXO-1 etc. Phosphorylation of these targets result in increased glucose uptake and glycogen synthesis as well as decreased gluconeogenesis.

Another major pathway for insulin signaling is the MAP kinase pathway. Insulin activates IRS, Gab1 and Shc, allowing them to recruit Grb2. Grb2 can bring SOS to the plasma membrane, where it activates G protein signaling cascade: Ras-Raf-MEK-ERK. Different from PI3K/AKT pathway, this G protein signaling is more associated with cell proliferation and differentiation effect of insulin [3].

O-GlcNAcylation is directly involved in diabetes and insulin resistance [4]. Overexpression of OGT in muscle, fat and liver leads to insulin resistance and hyperleptinemia [5]. Recent research described a novel phosphoinositide-binding domain in OGT. Under insulin stimulation, OGT translocates from nucleus to the plasma membrane, and specifically binds with PIP3. When OGT resides at the cell membrane, a cascade of phosphorylation signaling is regulated and O-GlcNAc attenuates insulin signaling [6]. Additionally, many key insulin signaling proteins, such as IRS, PDK, AKT, GSK3b, are substrates of OGT [7]. Meanwhile, there remains much to be learned about the specific roles of O-GlcNAcylation in insulin resistance. 6-Acetamido-6-deoxy castanospermine (6-Ac-Cas), another potent and cell permeable inhibitor of OGA was shown to be unable induce insulin resistance in 3T3-L1 adipocytes [8]. Both OGA overexpression and OGT knockdown in 3T3-L1 cells failed to prevent or mitigate glucose/insulin-induced insulin resistance [9].

IRS1 and IRS2 are key targets of insulin receptor tyrosine kinase. As an upstream adaptor protein in insulin signaling, a Yin-Yang, site-dependent phosphorylation regulation mechanism has been revealed on IRS. Once tyrosine residues are phosphorylated, and act as binding sites for SH2 domain-containing proteins, IRS positively mediates the PI3K branch of insulin signaling, which is critical for the regulation of insulin's metabolic effects. On the other side, IRS also contains many Serine/Threonine residues, which are heavily phosphorylated even in basal conditions. Serine phosphorylation of IRS is associated with dampened insulin action and underlies one molecular mechanism of insulin resistance [10].

Besides heavy phosphorylation on IRS, it is also O-GlcNAcylated. The O-GlcNAcylation sites are mapped to be mostly located in the C-terminus and close to docking sites of SH2 domain-containing proteins. The inhibition of PI3K binding with IRS1 due to

the C-terminal O-GlcNAcylation represents an additional layer of Yin-Yang regulation of IRS1 as well as insulin action [11].

O-GlcNAcylation and phosphorylation regulate insulin signaling simultaneously. However, how these two PTMs are interacting with each other is still under investigation. The present study was conducted to further investigate how O-GlcNAcylation and phosphorylation are crosstalking with each other in insulin signaling as well as to determine how O-GlcNAcylation of IRS1 regulates insulin action. We found that upon insulin stimulation, simply increasing or decreasing O-GlcNAcylation levels in 3T3-L1 adipocytes activates different alternative downstream signaling pathways. The different activated signaling pathways thus result in different insulin actions. We also mapped two new O-GlcNAcylation sites on mouse IRS1, Ser 635 and Ser 1005. O-GlcNAcylation on Ser635 negatively regulates O-GlcNAcylation at the C-terminus of IRS1, and thus positively regulates the metabolic arm of the insulin signaling pathway.

EXPERIMENTAL PROCEDURES

Reagents – All chemical reagents were obtained commercially unless otherwise noted. Ac_4SGlcNAc was synthesized in laboratory via published protocol [12]. Recombinant insulin was purchased from Sigma and TNFα was from Invitrogen. Antigen purified CTD 110.6 acites were purchased from antibody facility at University of Alabama, Birmingham, and then purified through a GlcNAc-Agarose column. Antibodies used in this project were against IRS1 (Millipore), myc (Santa Cruz), PI3K p85, phosphoThr308AKT, phosphoSer473AKT, AKT, phosphoThr202/Tyr204-ERK1/2, ERK1/2, phosphoSer21/9-GSK3ab, GSK3ab (Cell Signaling), actin (Sigma). Horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG, anti-rabbit IgG, anti-mouse IgM) were from Sigma. Blots were developed with ECL reagent (Denville Scientific) and Hyper-film (GE-Amersham Biosciences).

Cell Culture – 3T3-L1 preadipocytes were grown and differentiated into adipocytes as described [13] with slight modifications. The 3T3-L1 preadipocytes (within passage 3-10) were cultured in DMEM (4.5 g/L or 25mM glucose, Invitrogen) with 10% FBS (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin (Corning). Cells were kept at 37°C incubator with 5% CO_2 until reaching confluence (Day -2). 48 h later (Day 0), DEME culture medium with FBS and P/S were changed with 0.33μM DEX, 0.5mM IBMX, 1μg/ml insulin and 2μM rosiglitazone (Cayman Chemical) added freshly. The cells were kept in the differentiation medium for another 3 days (Day 3). After this, cells were incubated in DMEM culture medium containing FBS, P/S with1μg/ml freshly added insulin for 2 days (Day 5).

The medium was changed every 2 days afterwards. The differentiated 3T3-L1 cells were used for experiments within Day 9 ~ Day 11. For undifferentiated 3T3-L1 cells, very diluted status was maintained to avoid cell-cell contact.

CHO/IR and CHO/IR/IRS1 were kindly provided by Dr. Morris White laboratory (Harvard University), and the cells were grown with F-12 media supplemented with 10% FBS and 1xP/S. 293T cells were grown in DMEM media with 10% FBS and 1xP/S.

OGT and OGA inhibition on 3T3-L1 – 3T3-L1 cells seeded on 24-well plate were differentiated in DMEM containing 25mM glucose and 10% FBS, 1x P/S. At Day 9 the differentiated 3T3-L1 cells were incubated with 4 different culture media: 1) DMEM -5mM glucose 2) DMEM -5mM glucose with 0.6nM insulin 3) DMEM -25mM glucose 4) DMEM -25mM glucose with 0.6nM insulin plus 1% FBS, 1x P/S for 18 hours. In each culture media, we had 4 different treatments to regulate cellular O-GlcNAcylation levels: 1) no treatment 2) 1 μM TMG 3) 50 μM Ac-5SGlcNAc (AcSG) 4) 100 μM PUGNAc. So in total, we have 16 different culture and treatment conditions for differentiated 3T3-L1 cells. They were then serum starved and insulin deprived for 2h with a similar media rather than no FBS. After washing with Krebs-Ringer-Bicarbonate-HEPES (KRBH, 25mM HEPES, pH 7.6, 120mM NaCl, 4.6mM KCl, 1.9mM $CaCl_2$, 1mM $MgSO_4$, 1.2mM KH_2PO_4) buffer, the cells were cultured in KRBH for 10 min and then stimulated with 100nM insulin for another 15 min. The cells were harvested and cell lysate were collected immediately for later western blotting analysis.

Animals – All animals were processed following animal safety protocol according to Johns Hopkins University School of Medicine. 2-3 weeks old male (250g) Sprague Dawley rats were purchased from Harlan Laboratories and divided into 2 groups. After acclimation, they were randomly assigned into normal diet (Control, n=3) and high fat diet group (HF, n=3). After fed for 12 weeks, the rats were harvested and the abdominal fat pad tissue were collected and prepared for IRS1 immunoprecipitation analysis.

Plasmid constructs and protein overexpression – Constructs encoding myc-tagged mouse IRS1 as well as its control construct were kindly provided by Dr. Yehiel Zick laboratory (Weizmann Institute, Israel). Construct encoding recombinant full-length human OGT (ncOGT) was a gift from Dr. Suzanne Walker laboratory (Harvard University). For IRS1 overexpression, 293T cells, seeded at 1x 10^6 cells on 10-cm dishes the day before, were transfected using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen). Fresh DEME media were changed 12 h later and the cells with overexpressed mouse IRS1 were harvested for the following experiments 48 hours later.

For constructs with Ser635A and Ser635E site mutation, the following primer pairs were used: 1) Ser635A: 5' G CCC ATG AGC CCC AAG GCT GTA TCT GCC CCA CAG CAG- 3' and 5'- CTG CTG TGG GGC AGA TAC AGC CTT GGG GCT CAT GGG C-3' 2) Ser635E: 5'- G CCC ATG AGC CCC AAG GAA GTA TCT GCC CCA CAG CAG -3' and 5'- CTG CTG TGG GGC AGA TAC TTC CTT GGG GCT CAT GGG C-3'. PCR was conducted with using pfu Turbo (Agilent) with slightly modified procotol from QuickChange Lighting Site-Directed Mutagenesis (Stratagene). Briefly, 20 cycles of 95°C 30 s-65°C 30 s-72°C 1min/kb PCR reaction were performed first, followed with 2 h DpnI

(NEB) incubation at 37°C to cut the wild type construct template. Cell transformation was done using XL-10 Gold competent cell with β-ME (Stratagene). Positive colonies were selected and sequenced by sequencing facility at Johns Hopkins University.

For mIRS1 1-900 truncations, primer pair 5'-GGAGAATATGTGAATATTGAATTCTAGAGCGGCCAGCCTGG-3' and 5'-CCAGGCTGGCCGCTCTAGAATTCAATATTCACATATTCTCC-3' was designed for PCR. Similar protocol was used compared with Ser635A/E site mutagenesis rather than using KOD DNA polymerase (Millipore).

Western blotting and immunoprecipitation – Cells were washed with cold PBS for 3 times and then harvested with NP-40 lysis buffer (1% NP-40, 20mM Tris-HCl, pH8.0, 150mM NaCl, 1mM EDTA) plus various inhibitors (1μg/ml leupeptin, 2μg/ml antipain, 10μg/ml benzamidine, 10U/ml aprotini, 1mM PMSF, 2.5mM sodium orthovanadate, 10mM sodium fluoride, 10mM β-glycerophsphoate, 1μM TMG and 10μM PUGNAc). The whole cell extract was either sonicated 3 x 10s with a 30s pause on ice or incubated on ice for 30 min with vortexing every 10 min. The final cell lysate was collected through spinning the whole cell extract at 16,000 g for 20 min at 4°C. The lysate protein concentration was measured with Bio-Rad protein assay.

For 3T3-L1 cells differentiated within 24-well plate, they were directly lysed with pre-warmed 6x SDS loading buffer (0.35M Tris-HCl, pH 6.8, 10.28% SDS (v/v), 36% glycerol, 0.012% bromophenol blue (w/v), 5% β-mercaptoethanol). With the whole cell layer extracted into the loading buffer, the whole cell extract were transferred into 1.5ml microtube and boiled at 100°C for 10 min to break DNA in nucleus.

As for immunoprecipitation, 1mg cell lysate was precleared with control IgG and 10 µl pre-washed dynabeads® magnetic beads (Invitrogen) for 30 min at 4°C on rotator. 0.1% lysate were saved as input and the rest were incubated with targeting antibody overnight at 4°C on rotator. Next day, 20 µl magnetic beads were added into the lysate-antibody mixture for another 1 h incubation. The magnetic beads were then washed with NP-40 lysis buffer for 6 times with mild vortex each time. After wash, the beads were mixed with 6x SDS loading buffer, and boiled at 100°C for 5 min.

Pre-cast Criterion SDS-PAGE gels (Bio-Rad) were used to resolve the proteins, which were transferred to nitrocellular membrane (Bio-Rad). The membrane was blocked with 3% BSA (w/v, Sigma) or 5% milk (Safeway) in 0.1% Tween-20 TBST, and immunoblotted with indicated primary antibodies overnight at 4°C. After 3 times of 10 min wash with TBST on the next day, the membrane was incubated with HRP conjugated secondary antibody for 2 hours at room temperature followed with 3 times 10 min TBST wash and signal detection by ECL reaction.

Two-dimensional gel electrophoresis – One volume of 1mg CHO/IR and CHO/IR/IRS1 cell lysate were mixed with 4 volumn of 1:1 (v/v) 1mM HCl acidified acetone and methanol followed with overnight incubation at -20°C. The mixture was then spun at 15,000g for 15 min at 4°C. After carefully removing all the solvents, the protein pellets were air dried to eliminate acetone and methanol. 200 µl rehydration buffers (8M Urea, 2% CHAPS, 50mM DTT, 0.2% Ampholytes, 0.002% Bromophenol Blue) were added to resolve the protein pellet. The dissolved protein samples were loaded underneath the 11cm 3-10 ReadyStrip^{TM} IPG strips (Bio-Rad), and covered with mineral oil for a 12-14 h sample

absorption at RT. A 4-5 h isoelectric focusing was later carried out on Ettan IPGphor3 Dedcated IEF system (GE Healthcare) with recommended protocol. After equilibration to resolubilize proteins and reduce disulfide bonds, the second dimensional electrophoresis was performed with 10% SDS-PAGE. CHO/IR/IRS1 cell lysate was used as positive control for the following IRS1 western blotting detection.

Glucose uptake assay – Differentiated 3T3-L1 cells at Day 9 in 24-well plates were changed with various treatment media including 10% FBS for 18 h. For serum starvation, the cells were rinsed 3 times with PBS and incubated in a 0.5% FBS, 25mM HEPES treatment media for another 2 h. After being washed with freshly made KRBH buffer for 3 times, the cells were incubated with 0.1% FBS in KRBH for 20 min. 100nM insulin, 0.1 N HCl control as well as 20 μM cytochalasin B (Sigma) control were added into the incubation media for another 10 min to perform insulin stimulation followed with 0.5 μCi 1-[^{3}H]-2-DG (PerkinElmer) added into the media for another 5 min incubation. To remove the excess glucose and halt the uptake process, the cells were washed with cold PBS for 3 times, and immediately lysed with 0.4ml 1% SDS for 10 min at RT on shaker to accelerate extracting the cell layers into the lysis buffer. The whole cell lysate were then transferred into 4 ml scintillant and 1-[^{3}H]-2-DG uptake was measured using Beckman LS650 scintillation counter. Nonspecific deoxyglucose uptake was measured in the presence of cytochalasin B and was subtracted from the total glucose uptake readout.

In vitro ncOGT labeling – Recombinant ncOGT was purified as previously described and saved as 20% glycerol stock at -20°C [14]. Immunoprecipitated mIRS1-beads from

293T cells were mixed with 1 µl 1 Unit/µl Calf intestinal alkaline phosphatase (CIP, NEB), 1 µg ncOGT, 1 µl 200mM UDP-GlcNAc and 50mM HEPES, pH 7.6 to make 20 µl reaction mixture. The reaction mixture was then placed on rotator for 2 h incubation at RT. Reaction was terminated by washing the beads with 1% NP-40 buffer once to remove the excess OGT and CIP. 20ul 6x SDS loading buffer was added later to extract the ncOGT labeled mIRS1 from magnetic beads for SDS-PAGE analysis.

Peptide digestion and LC-MS/MS - ncOGT labeled mIRS1 samples were resolved by SDS-PAGE and stained with Coomassie G-250. mIRS1 bands were collected and in-gel digested with previously published protocol [15] . The bands were sliced into tiny pieces and destained by 25mM $(NH_4)HCO_3$ in 70% acetonitrile (ACN) , and then reduced by 0.1 M DTT, followed by 55mM iodoactamide reaction. The reduced mIRS1 protein was later digested with trypsin (Promega) overnight. The digested peptides were extracted from the sliced gels by repeating treatments with 5% formic acid ad 50% acetonitrile. All the extractions were combined into one tube and dried down under a speed vacuum. The peptides were then cleaned up by C18 spin column (Nest Group) to remove the remaining salts.

The salt-free peptides were separated into 2 parts, and analyzed by both CID and ETD fragmentation modes in mass spectrometer. The mass spectrometer was linked with an online auto sampler and a reverse-phase nano-2D liquid chromatography (LC) system (Eksigent Technologies). The LTQ-orbitrap Velos mass spectrometer was operated to capture both precursor and daughter ions with high resolution (Thermo Scientific). The LTQ

was programmed to record a full precursor scan (m/z: 350-1800) followed by fragmentation and MS/MS scans of the top 8 daughter ions in intensity.

RESULTS

OGT and OGA inhibitor treatment induce negative feedback regulation on the enzymes –Differentiated 3T3-L1 cells were treated with OGT or OGA inhibitors. First of all, the treatment condition was optimized and the treatment effects were confirmed by SDS-PAGE western blotting using pan specific O-GlcNAc antibody. We incubated differentiated 3T3-L1 cells in 4 different groups of culture media. Each group of cells was treated with control, specific OGA inhibitor TMG, specific OGT inhibitor AcSG and unspecific OGA inhibitor PUGNAc, which not only inhibits OGA, but also inhibits lysosomal hexoaminidases. We observed the obvious changes in cellular O-GlcNAcylation under OGT and OGA inhibition (Figure 6). We also observed increased OGT expression when OGT was inhibited, and elevated OGA expression when OGA was inhibited in all four media conditions. Through this negative feedback regulation on OGT and OGA protein expression, the stress caused by dramatic changes in cellular O-GlcNAcylation can be partially diminished. O-GlcNAc cylcling is highly involved in epigenetic regulation of gene expression as well as regulation of protein expression, via targeting various transcriptional factors, histones, ribosomal proteins and proteasomes [16]. As for the detailed mechanism of elevated OGT and OGA expression, more investigation needs to be done.

OGT and OGA inhibitors activate different branches of the insulin signaling pathways in 3T3-L1 cells – By altering cellular O-GlcNAcylation via inhibiting O-GlcNAc cycling in 3T3-L1 cells, we studied the crosstalk of O-GlcNAcylation and phosphorylation within insulin signaling pathway. With more than ten substrates identified for insulin

receptor tyrosine kinase, insulin impacts not only glucose, protein and lipids metabolism but also cell proliferation and differentiation. Activation of the INS/IRS/PI3K/AKT signaling cascade increases the translocation of GLUT4 to the plasma membrane and accelerates glucose uptake. In contrast, activation of INS/IRS or Shc/Grb2/RAS/RAF/ERK arms of the pathway leads to the proliferative effects of insulin, but with less impact on insulin's anabolic effects. For the cell lysates collected in the various inhibitor treatments, we measured site-specific phosphorylation on AKT, ERK and GSK3αβ by western blotting.

Phosphorylation of Threonine 308 and Serine 473 are required for full activity of AKT. Phosphorylation of Threonine 308 is the key requirement for the kinase activation. Inhibition of OGT activity by AcSG boosted phosphorylation of Thr308 but not Ser473 on AKT under insulin stimulation (Figure 6). The phosphorylation boosting effect was consistent under all four media conditions (DMEM with 5mM and 25mM glucose, with and without 0.6nM insulin). Co-incubation of 0.6nM insulin has been reported to gradually dampen insulin signaling in 3T3-L1 cells [9]. In our experiments, this effect was not obvious in cells grown in 5mM glucose. However, with high (25mM) glucose pre-incubation, in which 3T3-L1 showed relatively higher AKT activity, adding 0.6nM insulin did decrease phosphorylation of Thr308 on AKT and impair AKT activation. On the other hand, inhibition of OGA activity by TMG and PUGNAc did not cause significant difference on AKT activity compared with control group. This observation suggested the complexity of the crosstalk of O-GlcNAcylation and phosphorylation. Even though inhibition of OGT and OGA activity caused opposite affects on overall O-GlcNAcylation in 3T3-L1 cells, the affects on specific protein is not simply the opposite.

ERK1 and ERK2 regulate gene expression through phosphorylating downstream kinases as well as transcription factors; they also induce tRNA and rRNA synthesis to regulate protein expression. ERK1 and ERK2 share 90% sequence identity and both are expressed in adipocytes. Phosphrylation at both Threonine 202 and Tyrosine 204 sites on ERK1 and Threonine 185 and Tyrosine 187 on ERK2 are required for significant ERK activation with tyrosine residue proceeding threonine. Using the same 3T3-L1 cell lysate, we tested the activity of ERK1/2 through SDS-PAGE and blotted with site-specific phosphorylation antibody (Figure 6). Compared with no effects on ERK activity under AcSG pretreatment, specific inhibition of OGA by TMG treatment resulted in higher ERK activity under insulin stimulation. Our result provided a possible molecular mechanism to a previous report about inhibition of OGA does not induce insulin resistance in 3T3-L1 adipocytes [8]. Meanwhile, glucose concentration as well as physiological amount (0.6nM) of insulin in the incubation media did not affect ERK activity, as we expected.

As a downstream signaling molecule of AKT, GSK3αβ are phosphorylated and deactivated, thus stimulates glycogen synthesis by activating glycogen synthase. Considering the very low glycogen levels in adipose tissue, GSK3αβ is more associated with adipogenesis through phosphorylating transcription factor CCAAT/enhancer binding protein α (C/EBPα). Similar to previously report in neuroblastoma cells Neuro 2a [17], no change on GSK3αβ activity, as shown by using the specific antibody against GSK3αβ at Ser21/Ser9, was detected under enhanced AKT activity via AcSG inhibiting OGT (Figure 6).

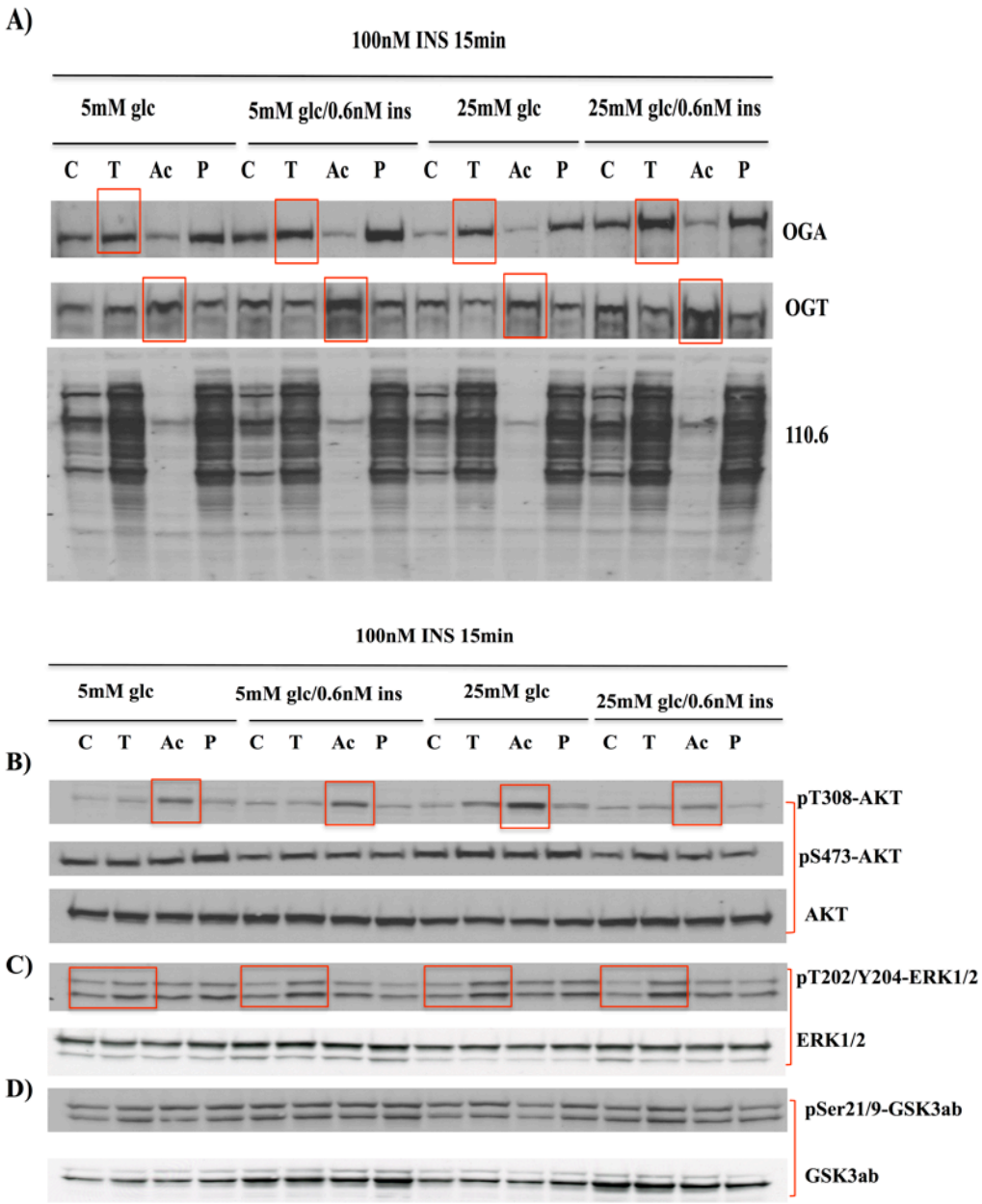

Figure 6. O-GlcNAcylation regulates unique insulin signaling pathways. Differentiated 3T3-L1 was switched to DMEM medium with low (5mM) or high (25mM) glucose, with or without physiological insulin (0.6nM). Different OGT or OGA inhibitors (C: control; T: TMG; Ac: AcSG; P: PUGNAc) were also added into the DMEM medium for coincubation. After serum starvation, the cells were stimulated with 100nM insulin for 15min. O-GlcNAcylation, OGT and OGA levels were detected (A.), different downstream signaling pathways under insulin were tested: Akt pathway (B.), ERK pathway (C.), GSK pathway (D.).

OGT inhibitor AcSG pretreatment enhances AKT signaling under insulin resistance - With the observation of OGT inhibition resulted in elevated AKT activity in 3T3-L1 regardless of glucose concentration as well as the existence of physiological insulin, we decided to further test AKT activity under induced insulin resistance condition, which has a relative low insulin stimulated AKT activity. A number of studies have demonstrated that tumor necrosis factor α (TNFα) pretreatment causes insulin resistance via loss of IRS1 and GLUT4 expression, and enhancement of serine phosphorylation on IRS1 [18, 19]. Based on Figure 6, enhanced AKT activity by OGT inhibition was most striking with high glucose DMEM incubation. We thus adopted the same media condition for TNFα pretreatment. Fully differentiated 3T3-L1 cells were pretreated with 10ng/ml TNFα for 4 hours before changing to 1% FBS media and coincubation with 10ng/ml TNFα (Sigma) and 50μM AcSG coincubation for another 18 hours. The cells were then serum starved with FBS free media for 2 hours before equilibrated with KRBH buffer for another 20 min. 100nM insulin was used to stimulate the cells for 15 min, and the whole cell lysate was subjected for SDS-PAGE and western blot. In Figure 7, as expected, compared with control treatment, TNFα pretreatment resulted in lower IRS1 expression and low amount of Threonine 308 phosphorylated AKT. Even TNFα pretreatment caused dampend AKT activity, AcSG coincubation with TNFα still had the highest AKT activity among the 4 types of pretreatments (Control vs TMG vs AcSG vs PUGNAc). All the above results shown in Figure 6 and Figure 7 suggest that partial OGT inhibition results in improved insulin responsiveness and may be a possible candidate for treating insulin resistance as well as type II diabetes.

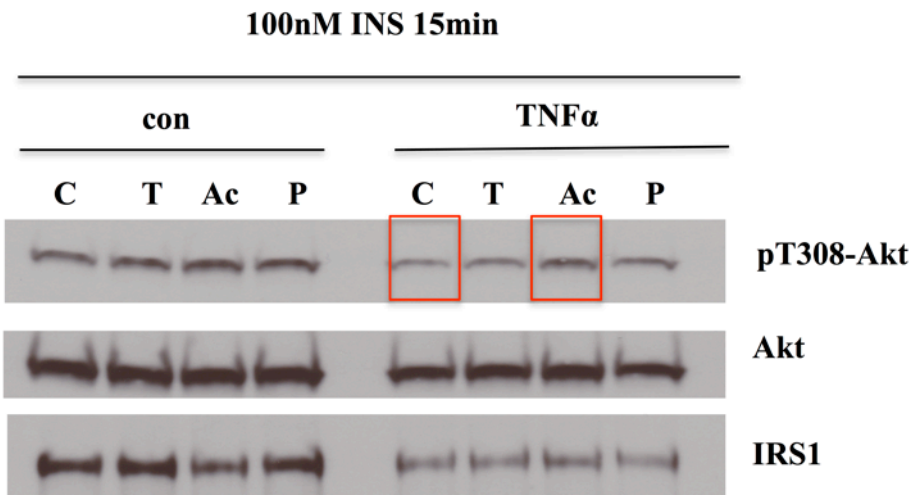

Figure 7. OGT inhibition boosted INS/IRS/AKT pathway under insulin resistance condition. WITH TNFα pretreatment, we mimicked insulin resistance on 3T3-L1 cell lines. The same OGT and OGA inhibition as Figure 6. were added into high glucose DMEM medium. The intensity of AKT signaling under insulin stimulation was detected through western blotting with anti-phospho Thr308-Akt antibody.

OGT inhibition greatly improved insulin stimulated glucose uptake in 3T3-L1 cells – In order to further demonstrate the possibility of using AcSG as a treatment for insulin resistance and Type II diabetes, we measured glucose uptake in 3T3-L1 cells under different treatments and media incubation. 3T3-L1 cells were incubated in 4 different media and treated with 4 conditions. As for 1-[^{3}H]-2-DG uptake, the nonspecific glucose uptake measured through cytochalasin B treatment was subtracted to only compare the specific glucose uptake through glucose transporters. Figure 8 illustrated the specific glucose uptake in 3T3-L1 cells listed by treatment as well as by incubation media. Compared with basal state (cluster 1), acute 100nM insulin stimulation (cluster 2) significantly increased glucose uptake into the cells, no matter the glucose concentration or existence of low-dose insulin in the DMEM media. Notably, pretreatment with AcSG to inhibit OGT activity within cells resulted in even higher insulin-stimulated glucose uptake (cluster 3 and 4). In addition, adding 0.6nM insulin in DMEM with both 5mM glucose and 25mM glucose impaired the glucose uptake at both basal state and insulin stimulated state. However, with AcSG pretreatment, this dampened glucose uptake effect was not present with 5mM glucose DMEM incubation, but only observed under high glucose DMEM incubation. In Figure 6, 0.6nM insulin incubation caused more severe impairment on AKT activity. This may be why we only observed decreased glucose uptake in high glucose DMEM media rather than in low glucose DMEM media.

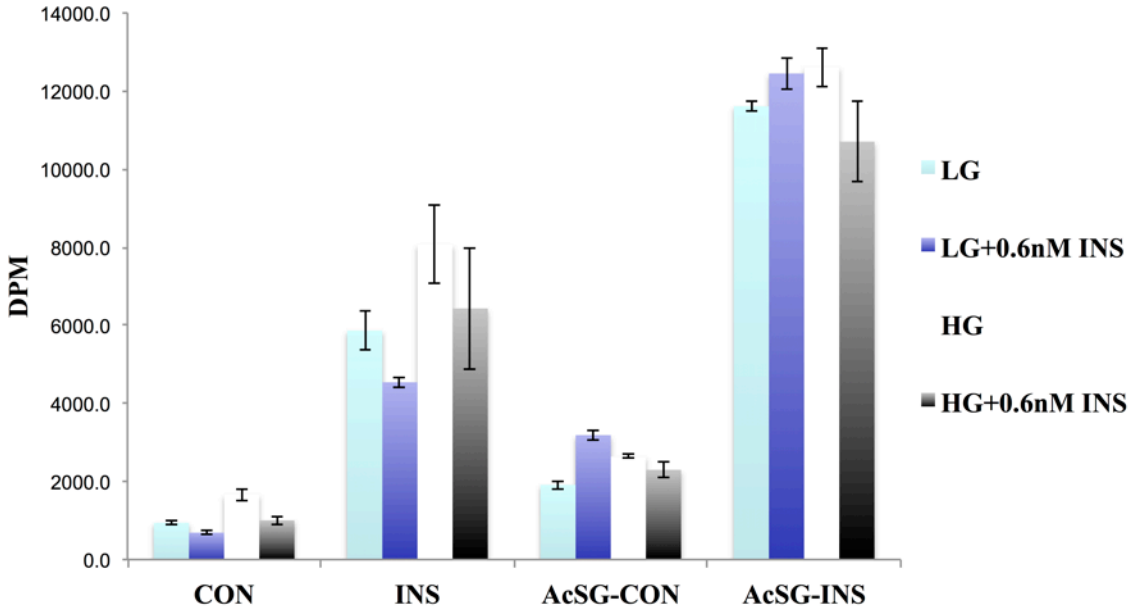

Figure 8. Glucose uptake assay under OGT or OGA inhibition. Same treatments as in Figure 6 were conducted on differentiated 3T3-L1 cell lines. Through chasing with radiolabelled unmetabolised 2-DG, the glucose uptake level was compared by the remaining radioactivity in the cells.

Elevated O-GlcNAcylated IRS1in adipose tissue of high fat diet fed rats – With observation of crosstalk of phosphorylation and O-GlcNAcylation in insulin signaling, we next studied the crosstalk between these two posttranslational modifications on specific protein IRS1. We found OGT and OGA inhibition boosted insulin stimulated AKT signaling and ERK signaling separately, which partially share the same upstream signaling molecule IRS. With OGT inhibition boosting insulin stimulated AKT signaling and glucose uptake, we further investigated the association of O-GlcNAcylated IRS1 with insulin resistance. For the past half century, a high fat diet has been proved to promote hyperglycemia and whole-body insulin resistance, and can be used to generate a valid rodent model for insulin resistance [20]. After feeding randomly grouped rats (n=3 for normal diet and n=3 for high fat diet) for 3 months, the abdominal fat pad tissue was collected for immunopreciptation of IRS1 and western blot with O-GlcNAcylation antibody 110.6. We found significantly elevated O-GlcNAcylated IRS1 on adipose in high fat diet fed group (Figure 9). Meanwhile we did not find any significant difference on overall O-GlcNAcylation in fat tissue between these two diets fed rats. Another research group has reported no changes of O-GlcNAcylation in the muscle and liver tissue of mice on a normal diet and high fat diet [21]. All these findings once again indicate the complexity between O-GlcNAcylation and insulin resistance. They also highlighted the vital regulation role protein-specific O-GlcNAcylation plays.

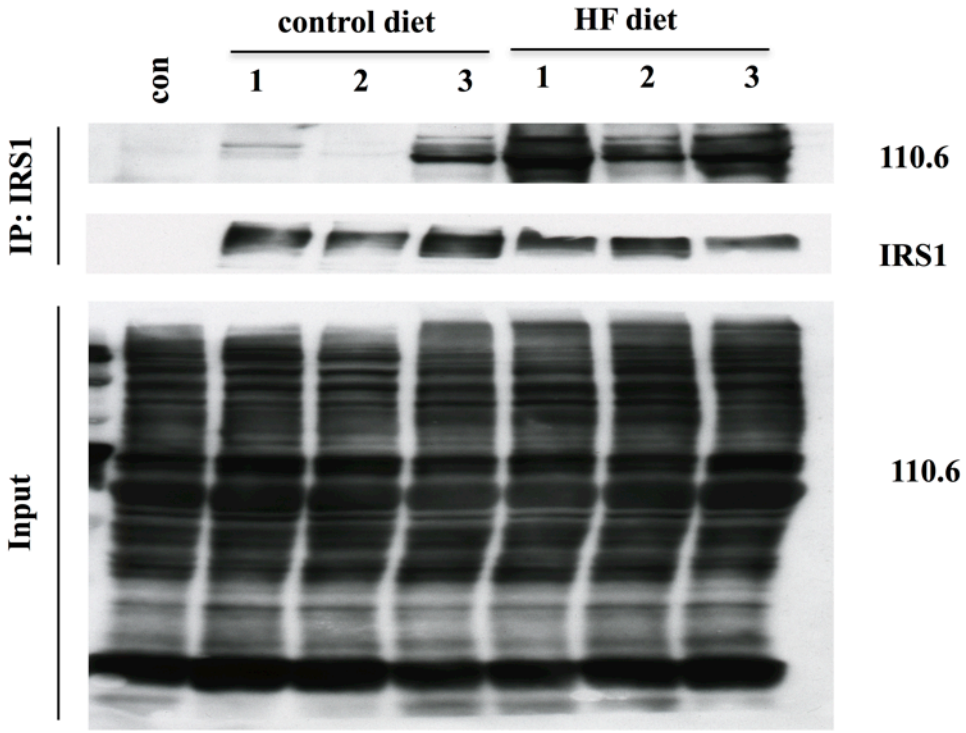

Figure 9. Elevated O-GlcNAcylated IRS1 is associated with HF-diet induced insulin resistance. 6 male SD rats were randomly assigned into control diet and HF diet group. After feeding with the diet for 3 months, the fat tissue was extracted and immunoprecipitated with IRS1 antibody. The O-GlcNAcylation level on IRS1 was then blotted with CTD 110.6 antibody.

Phosphorylation spectrum of IRS1 under OGT and OGA inhibition in CHO/IR/IRS1 cells – As an important upstream molecule in insulin signaling, IRS1 is exquisitely regulated through Yin –Yang phosphorylation. Serine phosphorylation serves as a negative regulator of insulin signaling while tyrosine phosphorylation on IRS1 positively regulates insulin signaling. Among all 1243 amino acids, IRS1 contains ~270 Ser/Thr/Tyr residues, many of which could be subjected to phosphorylation or O-GlcNAcylation. In order to acquire an overall view of the phosphorylation spectrum changes under OGT and OGT inhibition, we used two-dimensional electrophoresis (2DE) combined with western blotting. Affected by the high isoelectric point (pI) and molecular weight (MW) of IRS1, and low abundance of endogenous IRS1, we failed to detect any IRS1 stain from 3T3-L1 cells lysate even after numbers of optimizations. We later switched to CHO/IR/IRS1 cells, which have more concentrated IRS1 in the cell lysate [22]. Figure 10 showed the IRS1 blot of 2DE from different cell lysate. The further to the left a spot locates, the more phosphorylation it contains. Row 1 showed the IRS1 blot from CHO/IR cell lysate. With very low endogenous IRS1 expression in CHO cells, it can be served as a negative control for IRS1 blot. As expected, we did not observe any IRS1 spot at row 1. Row 2 to Row 5 are IRS1 blot results for CHO/IR/IRS1 cells under OGT or OGA inhibition. The wide spread of IRS1 stain on row 2 suggested the basal phosphorylation state of IRS1. With insulin stimulation (Row 3), we observed most of IRS1 spots moved towards the acidic side, which indicates hyperphosphorylation on IRS1. With TMG pretreatment and insulin stimulation, IRS1 was still under hyperphosphorylation. However, when comparing row 4 with row 3, we can tell IRS1 slightly transferred towards the basic end, except for a single hyperphosphorylation spot also found in basal state. OGT inhibition by AcSG induced a different phosphorylation

spectrum on IRS1 under insulin stimulation (Row 5). The IRS1 was shown as two clusters, one with hyper phosphorylation on the acidic side, the other is hypophosphorylation on the basic side. The unique phosphorylation spectrums on IRS1 under OGT and OGA inhibition demonstrated the extensive crosstalk of O-GlcNAcylation and phosphorylation once more, also indicated different downstream signaling will be activated.

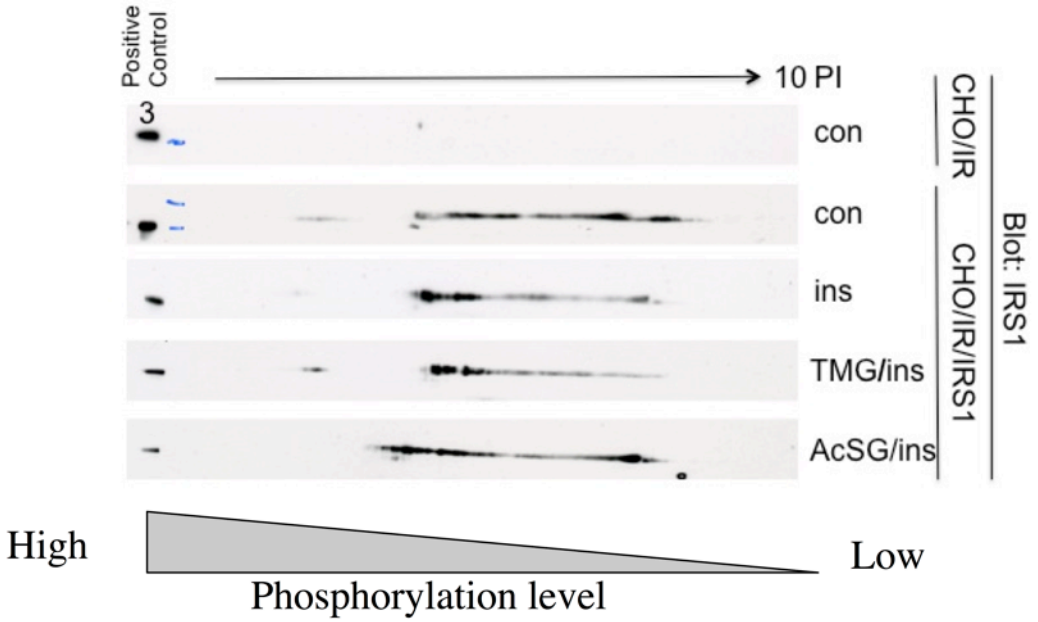

Figure 10. OGT and OGA inhibition caused different phosphorylation spectrum on IRS1. IRS1 overexpressed CHO/IR/IRS1 as well as the negative control CHO/IR cells were serum starved, and then treated with 100nM insulin for 15min. The total cell lysate were then precipitated to remove the ions. The pure protein pellet was then resolublized in equilibration buffer and absorbed into 11cm IPG strips. After running both dimensions, the protein sample was blotted with IRS1 antibody. The lefter a spot locate, the higher phosphorylation it indicates.

O-GlcNAcylation of mouse IRS1 at Ser 635 and Ser 1005– With several O-GlcNAcylation sites have been mapped on the C-terminus of rat and human IRS1, we decided to perform additional site mapping on mouse IRS1. One goal was to test whether the O-GlcNAcylation sites were conservative among species; the other one was to find more O-GlcNAcylation sites. We overexpressed myc-tagged mouse IRS1 on 293T, and pulled down the IRS1 by anti-myc antibody. Figure 11 showed O-GlcNAcylation of overexpressed IRS1 in 293T. By inhibiting O-GlcNAc cycling through TMG, O-GlcNAcylation of IRS1 has been increased. We also incubated the immunoprecipitated IRS1 with *Clostridium perfringens* NagJ (CpNagJ) *in vitro*, a close homologue to human OGA. The disappeared O-GlcNAcylated IRS1 band proved our O-GlcNAcylation blot by the antibody CTD 110.6 was very specific.

In order to increase the stoichiometry of O-GlcNAcylation on the IRS1 sample, besides treating 293T cells with TMG, we in addition *in vitro* OGT labeled the pulled down IRS1. Figure 11 was the O-GlcNAcylation blot for an aliquot of the IRS1 sample sending for site mapping. Compared with the left lane, IRS1 labeled by OGT *in vitro* had elevated O-GlcNAcylation. The OGT labeled IRS1 sample was then run on SDS-PAGE, and followed with coomassie staining. All the IRS1 bands in SDS-PAGE was collected, trypsin digested and salt cleaned up. The digested peptides were sent for later mass spectrometry site mapping by Dr. Akilesh Pandey laboratory.

Combing CID and ETD MS/MS, we found 2 new O-GlcNAclation sites on mouse IRS1, Ser 635 and Ser 1005. Ser 1005 site has also been demonstrated with O-GlcNAcylation on rat and human sample [11]. Figure 11 represented the MS/MS

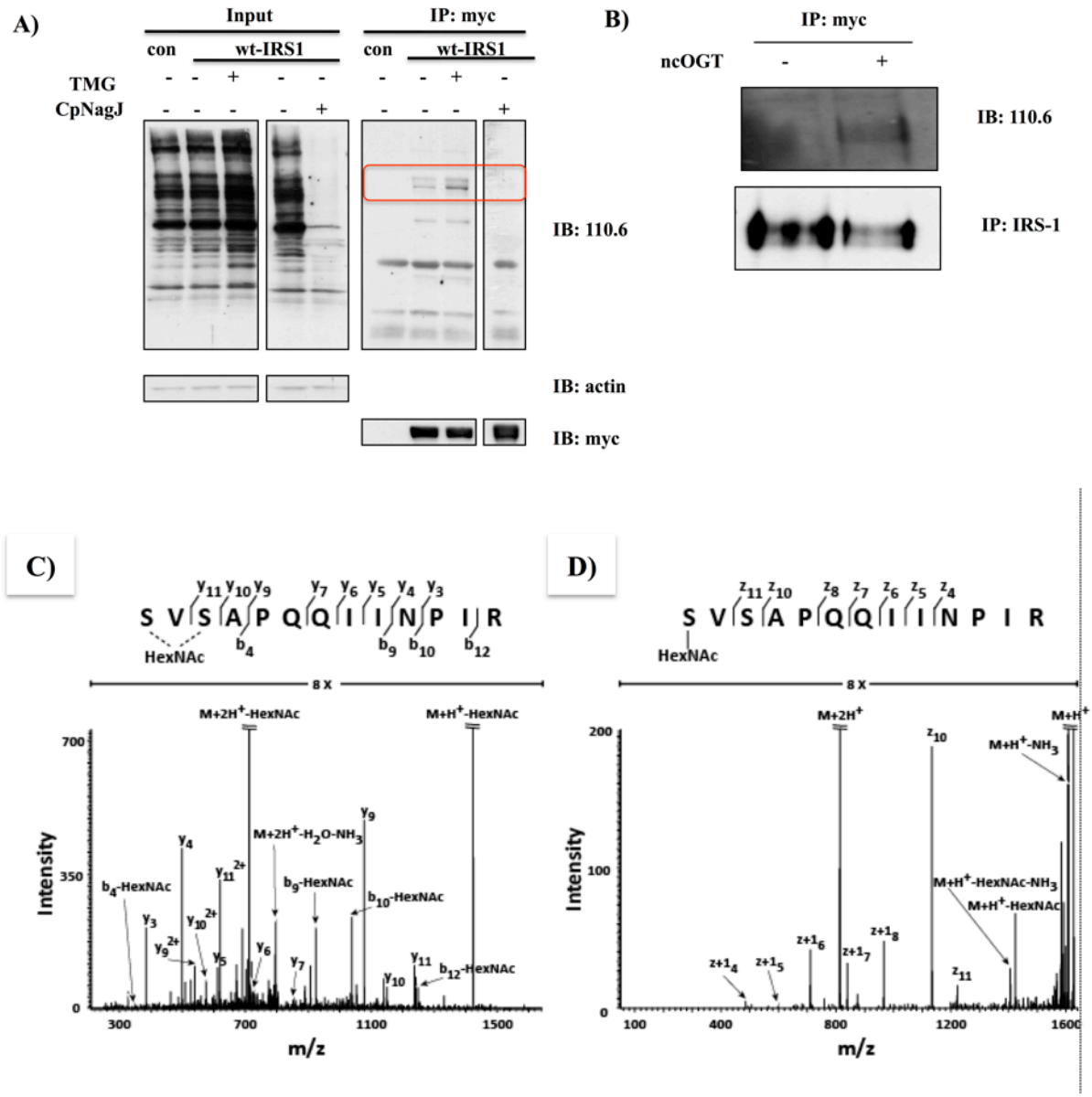

Figure 11. O-GlcNAc site mapping of mouse IRS1. Myc-tagged mouse IRS1 was overxpressed in 293T cells. TMG (A) and ncOGT labeling (B) increase the stoichiometry of O-GlcNAcylation on IRS1 (C, D). The immunoprecipitated IRS1 sample from TMG treated 293T cells was sent for nc OGT *in vitro* labeling, and in gel digestion. The peptides were then analysed by CID (C) and ETD (D) for potential O-GlcNAcylation site. The MS spectrums of O-GlcNAcylation on Ser635 were shown here.

fragment spectrums of the precursor peptide containing Ser635. From CID MS/MS shown in Figure 11, we detected b4, b9, b10 and b10 fragment ions with one O-GlcNAc group, demonstrated O-GlcNAcy locating on either one or both of the serine residues. In Figure 11, precursor with one GlcNAc group and z11 fragment ion without O-GlcNAc group were observed, which suggested O-GlcNAc was located on Ser 635 rather than Ser 637.

OGT inhibition dampened phosphorylation of Ser632/635 on IRS1 – Besides the newly mapped O-GlcNAcylation on Ser635 of IRS1, phosphorylation on Ser 632/635 has been shown to play an important role in negatively regulating the metabolic effects of insulin. With two types of modification identified on Ser635, we were interested in the phosphorylation changes when we chemically change O-GlcNAcylation in 3T3-L1 cells. Figure 12 showed that phosphorylation and O-GlcNAcylation on IRS1 was not simply inhibitory to each other. OGA inhibition, causing elevated O-GlcNAcylation in cell lysate, did not affect phosphorylation of Ser632/635 significantly. However, OGT inhibition, with barely detected O-GlcNAcylation within cells, impaired phosphorylation of Ser632/635 on IRS1, which was a negative regulator of downstream AKT pathway. This finding was consistent with our findings in Figure 6: AcSG pretreatment rather than TMG pretreatment boosted insulin stimulated AKT pathway.

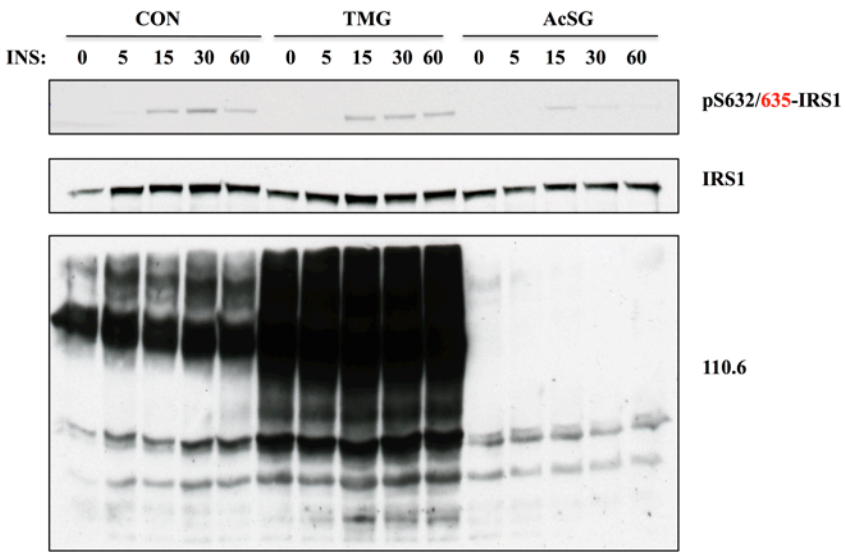

Figure 12. Phosphorylation cycling on Ser635 of IRS1 under OGT and OGA inhibition. Differentiated 3T3-L1 cells were serum starved in high glucose DMEM, and treated with control, TMG or AcSG before stimulated with 100nM insulin for the certain time course (0,5,15,30,60 min). The cell lysate were analyzed by specific phosphorylation antibody for Ser632/Ser635.

O-GlcNAcylation on Ser635 negatively regulate O-GlcNAcylation on the C-terminus of IRS1 – With the observation of decreased O-GlcNAcylation on Ser635 and decreased phosphorylation on Ser632/635 under OGT inhibition, we further investigated the function of O-GlcNAcylation of Ser635 on IRS1. Figure 13 showed the effect on IRS1 O-GlcNAcylation when mutated Ser635 to Ala or Glu. We were expecting a decreased or unchanged O-GlcNAcylation on site mutated IRS1, but observed increased O-GlcNAcylations on Ser 635A and Ser635E mutated IRS1. Ser635E-IRS1, mimics of phosphorylated IRS1 on Ser635-IRS1, facilitated O-GlcNAcylation on other sites. Ser635A-IRS1, with no O-GlcNAcylation or phosphorylation on this site, furtherly elevated O-GlcNAcylation on IRS1. Combing these two results together, we hypothesized that O-GlcNAcylation on Ser635-IRS1 may act as a negative regulator of O-GlcNAcylation on other sites. Considering all other currently identified O-GlcNAcylation sites are located on C-terminus, we next tested the crosstalk of O-GlcNAcylation on Ser635 and O-GlcNAcylation at the C-terminus.

We made a C-terminal truncated $IRS1_{1\text{-}900}$ constructs with Ser635, Ser635A and Ser635E. When comparing the O-GlcNAcylation of this truncated IRS1 and the full-length IRS1 (Figure 13), we found a dramatical decrease of O-GlcNAcylation on IRS1, suggesting the main O-GlcNAcylation sites on IRS1 locate at the C-terminus. The faint O-GlcNAcylation blot on Ser635A- $IRS1_{1\text{-}900}$ suggested other O-GlcNAcylation sites exist besides Ser635 on the truncated IRS1. We also reconfirmed O-GlcNAcylation on Ser635 in the truncated IRS1 form. With Ser 635 mutated into Ala and Glu, O-GlcNAcylation of truncated IRS1 decreased in the truncated IRS1 protein.

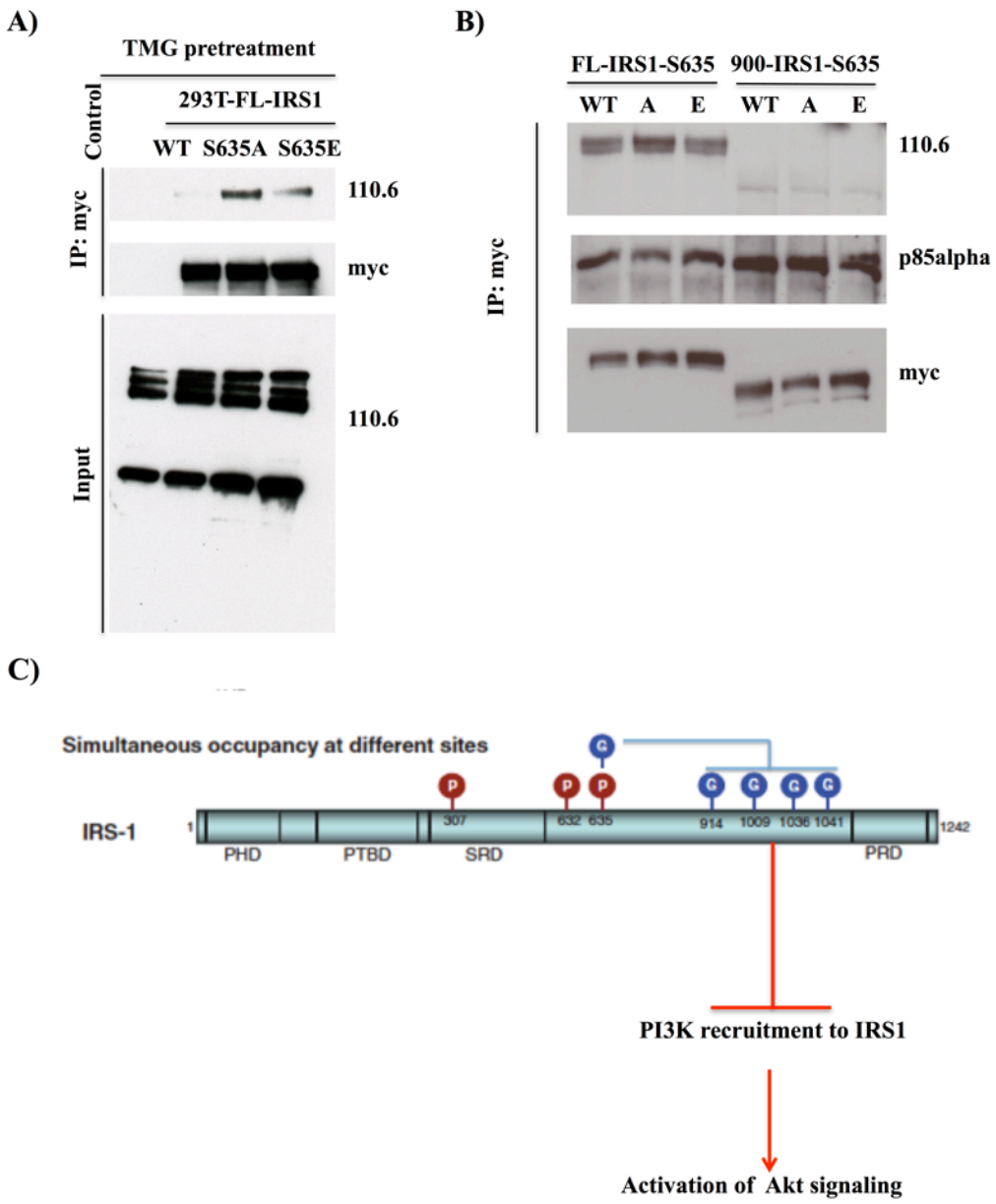

Figure 13. Crosstalk between O-GlcNAc on Ser635 and C-terminal O-GlcNAc residues on IRS1. (A) Total O-GlcNAcylation level on the overexpressed IRS1 in 293T cells were measured on the wild type (WT), Ser635A and Ser635E mutants. (B). A C-terminus truncated IRS1 (900-IRS1) was compared with full length (FL-IRS1) in the overall O-GlcNAcylation level, p85 alpha binding with IRS1 among WT, Ser635A and Ser635E mutants. (C). The proposed model of the function of O-GlcNAcylation on Ser635 of IRS1.

C-terminal O-GlcNAcylation is reported to inhibit nearby tyrosine phosphorylation, which act as the docking sites for PI3K. With C-terminus truncated, we detected the binding between $IRS1_{1\text{-}900}$ and PI3K. To our surprise, we found a much stronger binding between PI3K and the truncated $IRS1_{1\text{-}900}$. The reasons for this observation could be: first: tyrosine phosphorylation on $IRS1_{1\text{-}900}$ (such asY608, 628) are the main docking site for IRS1. The other would be with the overall effect of C-terminal O-GlcNAcylation and phosphorylation on IRS1 is inhibitory to PI3K binding. We then compared the PI3K binding with IRS1 among Ser635, Ser635A and Ser635E. With elevated overall O-GlcNAcylation on Ser635A-FL-IRS1, we observed decreased PI3K binding, consistent with the negative regulation of PI3K binding by the C-terminal O-GlcNAcylation. The decreased binding with PI3K through Ser635A mutation disappeared on $IRS1_{1\text{-}900}$, since in the truncated IRS1, O-GlcNAcylation on Ser635 lost its inhibitory crosstalk with the C-terminal O-GlcNAcylation.

Our model of O-GlcNAcylation on IRS1 is summarized in Figure 13, with extensive crosstalk between phosphorylation and O-GlcNAcylation, among O-GlcNAcylations and among phosphorylations that exist on IRS1.

DISCUSSION

O-GlcNAcylation and phosphorylation are two rapidly cycling posttranslational modifications both occuring on Serine and Threonine residues. Large-scale proteomics studies suggested O-GlcNAcylation and phosphorylation could function both synergistically and antagonistically [23]. Insulin stimulates phosphorylation on different signaling molecules, thus regulates various cellular processes, including glucose metabolism and cell growth. Disordered insulin signaling is highly associated with disease development, such as type II diabetes and cancer. Herein, we further explored the crosstalk of O-GlcNAcylation and phosphorylation in insulin signaling, mainly focused on how O-GlcNAcylation affects glucose metabolism related insulin signaling.

Many controversies exist on the association of O-GlcNAcylation with type II diabetes and insulin resistance [24]. Glucosamine treatment, GFAT and OGT overexpression caused insulin resistance. On the other hand, specific OGA inhibitor treatment, OGA overexpression and OGT knockdown did not cause insulin resistance in other models. In our research, we found OGT and OGA inhibition caused negative regulation on OGT and OGA protein expression, and revealed the difference between genetic and pharmacological regulation of O-GlcNAcylation. Our data showed OGT and OGA inhibition, respectively each boosted different insulin stimulated signaling cascades. The fact that OGA inhibition did not affect INS/IRS/AKT signaling pathway, is consistent with previous observation that a very specific OGA inhibitor did not induce insulin resistance.

The effect of OGT inhibition on 3T3-L1 cells has never been studied. With the development of an OGT inhibitor [12], we studied its pharmacological effect on 3T3-L1. We

demonstrated the potential for inhibitors like AcSG to ameliorate insulin resistance and type II diabetes. AcSG treatment of 3T3-L1 dramatically boosted insulin stimulated AKT signaling pathway, under various incubation conditions, such as low glucose, high glucose, +/- low-dose insulin in the culture media. Under insulin resistance induced by TNFα on 3T3-L1, AcSG's effect on the AKT signaling still occurred. Consistent with the effect of elevated AKT activity, AcSG also dramatically increased glucose uptake in 3T3-L1 cells. Our data systematically presented the potential usage of treating type II diabetes with OGT inhibitors, such as AcSG. Considering we only tested the effect of AcSG on adipocytes, more studies on muscle and liver and animals are required to evaluate the usefulness of OGT partial inhibition in treatment for diabetes.

We also showed the crosstalk of O-GlcNAcylation and phosphorylation on IRS1, which can be extensively phosphorylated on serine and tyrosine residues to regulate insulin signaling. With showing high fat diet induced insulin resistant rat had elevated O-GlcNAcylated IRS1 level on fat tissue, we were inspired to study the effect of O-GlcNAcylated IRS1 in the development of insulin resistance. With IRS1 located upstream of both AKT and ERK signaling pathways, and since OGT and OGA inhibition specifically boosted AKT and ERK activity, we first measured the phosphorylation status of IRS1 under OGT and OGA inhibition. 2DE blot revealed OGT and OGA pretreatments caused distinct phosphorylation patterns on IRS1, which may be linked with the activation of unique insulin stimulated signaling pathways. AcSG pretreatment induced a "two-clustered" phosphorylation pattern on IRS1 under insulin stimulation, which indicated hyperphosphorylation and hypophosphorylation coexist on IRS1. TMG pretreatment caused hyper phosphorylation on IRS1 under insulin stimulation, including an extreme

phosphorylation also shown in basal condition. More details about the insulin stimulated phosphorylation on IRS1 under OGT and OGA inhibition can be revealed through SILAC experiments. Clarified site-specific phosphorylation changes on IRS1 would help us figure out how phosphorylation on IRS1 regulates the recruitment of different downstream signaling molecules.

Previous literature showed O-GlcNAcylation occurred at the C-terminus of IRS1. Our research further confirmed this with mapping O-GlcNAcylation of Ser1005 on mouse IRS1as the homologous residues on rat and human IRS1. In addition, we found another O-GlcNAcylation on Ser635 residue, which was considered to be one of the most important phosphorylation sites in regulating insulin signaling. Depending on the upstream kinases, the phosphorylation on Ser635 has been indicated in either upregulating or downregulating insulin signaling. The mammalian target of rapamycin (mTOR)/p70S6K phosphorylates Ser632/635 residues and negatively regulate insulin signaling [25]. While Rho-kinase (ROK) phosphorylated Ser632/635 caused significantly increased insulin-stimulated tyrosine phosphorylation, IRS1 binding to p85 subunit of PI3K as well as IRS1 associated PI3K activity [26]. With only one enzyme regulating O-GlcNAcylation, we were interested in identifying the O-GlcNAcylation effect on Ser635 of IRS1.

Elevated O-GlcNAcylation on Ser635Ala-IRS1 indicated the crosstalk of O-GlcNAcylation among Ser635 and other potential sites. The crosstalk may happen through structure inhibition or competitive OGT recruitment. With limited information on IRS1 structure, it is hard for us to exclude either possibility. With all other documented O-GlcNAcylation sites located on C-terminus, we further tested and confirmed the crosstalk of O-GlcNAcylation between Ser635 and C-terminal sites through using C-terminus truncated

$IRS1_{1\text{-}900}$. The elevated O-GlcNAcylation on Ser635A-FL-IRS1 did disappear onSer635A-$IRS1_{1\text{-}900}$. As for IRS1 associated p85 binding, we found Ser635A mutation decreased the binding on full length IRS1 rather than C-terminus truncated IRS1. In addition, Ser635E mutation caused slight but not significant decrease of PI3K binding in both full length and truncated IRS1. In summary of the findings from site mutagenesis and protein truncation, we showed O-GlcNAcyaltion on Ser635 appears to negatively regulate O-GlcNAcylation on C-terminus of IRS1, thus positively regulating PI3K binding and downstream signaling.

Based on our study, we provided a general picture of how O-GlcNAcylation and phosphorylation interact with each other. Globally regulating O-GlcNAcylation level by OGT and OGA inhibition specifically regulated different insulin-stimulated signaling pathways, which may be explained by the various phosphorylation patterns on the signaling molecules. O-GlcNAcylation sites also interacts with each other on single protein, as shown in IRS1; O-GlcNAcylation on Ser635 acts as a positive regulator of insulin action through inhibiting other inhibitory O-GlcNAcylation. With phosphorylation also happening on the same residue, we discovered the elaborate regulation of signaling pathways through interaction between O-GlcNAcylation and phosphorylation.

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